ACTA Biologica

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TOMUS XV
FASCICULUS 1

AKADÉMIAI KIADÓ
BUDAPEST 1964

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A MAGYAR TUDOMÁNYOS AKADÉMIA BIOLÓGIAI KÖZLEMÉNYEI

SZERKESZTŐSÉG: BUDAPEST IX., TÜZOLTÓ U. 58. KIADÓHIVATAL: BUDAPEST V., ALKOTMÁNY U. 21.

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Abonnementspreis pro Band: 110 Forint. Bestellbar bei dem Buch- und Zeitungs-Außenhandels-Unternehmen »*Kultúra*« (Budapest I., Fő utca 32. Bankkonto No, 43-790-057-181) oder bei seinen Auslandsvertretungen und Kommissionären.

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TOMUS XV

AKADÉMIAI KIADÓ, BUDAPEST
1965

Acta Biol. Hung., **15** (1), 1—10 (1964)

STUDIES ON RESPIRATION DURING REGENERATIVE CHONDRAL BONE FORMATION (FORMATION OF CALLUS)

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(Received July 1, 1963)

Synopsis

The authors have studied the tissue respiration of the internal and external callus in 74 dogs, by means of the Warburg technique. In one group of dogs the fractures were treated by medullary nailing while in the other no nailing was employed. The studies were conducted 1, 2, 3, 4 and 6 weeks after fracture. In the medullary nailing group the external callus initially showed increased respiration, then after the third week respiration diminished. Grossly the same applies to the internal callus. In the no-nailing group the rise reaches the maximum at 3 weeks, there is a temporary reduction of respiration during the fourth week, and subsequently respiration tends to increase again during the sixth weeks. The internal callus shows an excessively high rate of respiration at 3 weeks, followed by a marked decrease during the fourth, then by another rise during the sixth week. On the basis of an analysis of the factors potentially influencing tissue respiration the authors arrive at the conclusion that the increased rate of tissue respiration is due to an impairment of tissue metabolism resulting from the disturbance of vascularisation following fracture, while a longer persistence, natural or artificial, of the noxa leads to a reduction in respiratory activity, a morphological manifestation in the extensive cartilage cell. As ossification is gaining preponderance, the respiratory rate shows a parallel increase. The evidence obtained tends to indicate that in the animals of the medullary nailing group the damage is more severe, as a result of which cartilage formation persists for a longer period.

Introduction

The metabolism of the normal ossifying cartilage cell has not been studied extensively and in detail. Lutwak-Mann [16], Bywaters [2, 3, 4], Hills [10] found more intense anaerobic glycolysis and very low oxygen consumption in the articular cartilage. Oláh and associates [20, 21] studied the respiration of the regenerative articular surface and found that respiration increased in the early phase of cartilage formation, then it decreased.

Hoffman and co-workers [11] have found no oxygen consumption at all in the epiphyseal cartilage. Picard and Cartier [22, 23] found a close correlation between glycolysis and cellular respiration or mineralisation, respectively. It seemed therefore proper to supplement our earlier results [6, 7, 8] with data on metabolism, i.e. to approach the problem of regenerative chondral bone formation not only from the morphological, but also from the bio-

chemical angle. Therefore we have undertaken to study the respiration of callus, to obtain evidence relative to the metabolic process taking place in the different phases of regenerative chondral bone formation.

Material and method

74 dogs of different breed were used, divided into two groups containing 37 animals each. In one group the animals were pretreated with morphine, anaesthetized with ether, the skin was incised over the left tibia, a hole was bored into the medullary cavity of the bone, a Küntscher nail was inserted, then the bone was sawn through, taking care, to leave the fibula intact. The medullary nail was pushed down, keeping thereby the fracture ends fixed, and finally the skin wound was sutured.

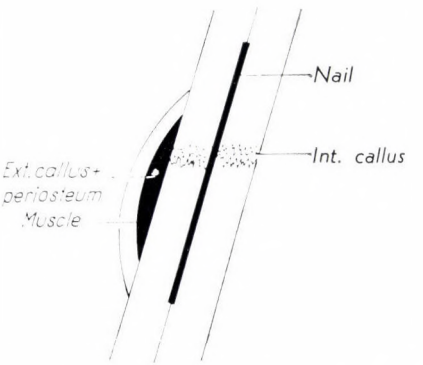

Fig. 1. Diagrammatic representation of the in situ topography of the external and internal callus tissues used for measuring respiration

In the other group the procedure was similar, except that no medullary nailing was employed. The operations were performed with strict asepsis, sterile bandages were placed over the sutures, and removed 3 to 4 days later. From one week before operation on until study the animals were kept under identical conditions and were fed the same diet.

The animals of both groups were sacrified 1, 2, 3, 4 and 6 weeks after operation, respectively, by hitting them on the nape and exsanguination. After that, the area of the fracture was excised about 5 cm above and 5 cm below the line of fracture, and the tissues to be studied were prepared out as fast as possible. The muscles and tendons superior to the site of fracture were cut off with a knife, and the external callus tissue was prepared off. Then we penetrated through the external callus in between the ends of the fracture and excised the central (internal) callus. In situ topography of the two parts is shown in Fig. 1. The specimens removed as fast as possible were placed into a dish chilled in ice water, minced finely by scissors, and 0.2 to 0.4 g amounts (measured by analytical scales) were added to the Warburg dish. Every step was carried out rapidly and possibly under chilling. The interval between killing the animal and the onset of study varied from 30 to 45 minutes. Then we studied respiration at 37° C according to WARBURG [27]. To the minced tissue we added 0.5 ml of a 1 per cent glucose solution and sufficient Krebs Ringer phosphate buffer to adjust the volume of 3.2 ml, the volume taken into consideration when the constants of the vessels were calibrated, including the volume of the specimen added and the 0.2 ml 20 per cent KOH in the centre well of the Warburg apparatus. When calculating its volume, the specific gravity of the tissue studied was considered to be 1. The manometers of the "Labor" Warburg apparatus were shaken at a rate of 70/min., first with the stopcocks open, then when it assumed the temperature of the water bath, the stopcocks were shut. Thus, the specimens were exposed to atmospheric air.

After 4 hours of respiration the Warburg vessels were taken down, the contents were washed into the crystallizer bulbs, wherein they were dried to constant weight and weighed for dry solids. The results obtained are related to this dry weight.

Mincing the specimens represented the greatest difficulty in the procedure, because the small quantity of the material available, and mainly the fact that it was difficult to mince, made it impossible to use either the Potter homogenator, or Latapie's grinder, or the Waring blendor. The only possible way to mince the material was to use scissors, and mincing was continued until the pieces were so small that the scissors could not catch them any more.

The subjectivity of assessing the measure of mincing and the fact that the material was worked in air and not in O_2 atmosphere explain why we did not calculate the $Q \cdot O_2$, either. The data obtained can be used for comparing the results obtained for the single layers of the callus and for the determination of the differences in the results measured at different points of time after the fracture.

The specimens were studied histologically, too, in order to determine whether the regeneration of bone had proceeded according to the normal patterns.

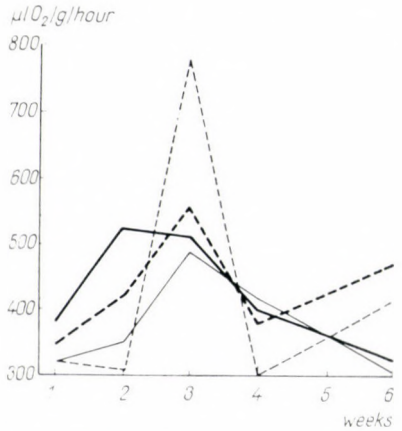

Fig. 2. Diagrammatic representation of the average respiration values of the external and internal callus in dogs with and without medullary nailing
——— external and ..... internal callus with nailing; — — — and — — — controls

Results

The results of the respiration studies, as well as on the basis of the average values the respiratory rates of the external and internal calluses of the two groups in the different stages have been represented graphically (Fig. 2.) The data reveal that in the medullary nailing group one week after operation the external callus consumed 360 microlitres of oxygen in 1 hour. This, as well as the other numerical data express the oxygen consumption by 1 g dry solids in 1 hour, in microlitres. During the second week the external callus consumes even more oxygen, then oxygen consumption gradually decreases from the third week on until the sixth week, to 329 microlitres. In the medullary nailing group the oxygen consumption of the internal callus was 319 microlitres during the first week, followed by a slight decrease during the second, and a rise during the third; finally, the decrease continued during the fourth and sixth weeks (to 309 microlitres), like in the case of the external callus. In the no medullary nailing group the external callus consumed 341 microlitres of

oxygen by the end of the first week. Then oxygen consumption increased up to 549 microlitres until the third week, followed by a minimal decrease during the fourth, then another increase during the sixth. The internal callus showed rather irregular respiration values. During the first week the oxygen consumption was 321 microlitres, followed by a decrease during the second and a remarkable increase during the third week, then during the fourth week it fell steeply back to the first week value, and finally there came a rise again during the sixth week.

Comparison of the respiration values of the external calluses of the two groups reveals that oxygen consumption is higher during the first two weeks in the medullary nailing group, whereas in the no-nailing group the oxygen consumption is higher in the rest of the observation period. As to the internal calluses, the nailed group showed higher oxygen consumption in the first two weeks, during the third week oxygen consumption was higher in the not nailed group, during the fourth the nailed, and during the sixth the not nailed showed higher oxygen consumption values. It is to be noted that the levels at which the differences were significant were: 6-week external calluses 0.02, 3- and 4-week control internal calluses 0.05, 3-week nailed and control internal calluses 0.10, 3- and 4-week nailed external calluses 0.10, 3- and 4-week control external calluses 0.10, and 6-week internal calluses 0.116. In the other stages the difference between the respiratory values of the nailed and control animals was not significant. The significance was calculated by the Wilcoxon test.

Discussion

The results obtained indicate that in the case of the external calluses, in the first place, the respiratory values are higher in the nailed animals during the first week already, and after reaching a maximum in the two-week period, they continue to decrease gradually till the end of the observation period. As opposed to this, in the non-nailed (control) dogs the maximum respiration is found in the third week, then comes a decrease lasting about 1 week, followed by a rise again. In the nailed animals the external calluses show higher respiratory rates during the first three weeks than the internal ones, after that the differences are levelled out. Except for the 3-week values, in the nailed animals the oxygen comsumption by the internal callus was lower than that by the external callus. As to the nailed and control animals, the oxygen consumption of the internal callus was slightly higher in the controls during the first two weeks and during the fourth week.

What then accounts for the differences and what factors may be involved in the changes of tissue respiration.

1. Vascularisation should be thought of, in the first place. It appears to be natural that the vascular supply to the tissues and cells has a significant

role to play in their respiration, because it is by way of the blood vessels that nourishment and oxygen are supplied to the tissues and cells. In this connexion attention should be directed to our earlier work [15], in which we had examined the vascularisation of the callus in nailed and non-nailed animals at different intervals, injecting China ink and employing the Spalteholtz method. It was found that after fracture capillarisation begins periosteally during the first week, there is no activity in the endosteum, while at the same time in the medullary nailed animals a minimum amount of cartilage tissue is found under the periosteum. During the second week the vascularisation from the periosteum is arrested for some reason, correspondingly the islet of cartilage increases in size; endosteally, the blood vessels continue to be absent. During the third week endosteal vascularisation starts; the periosteal capillaries too, show more and more ramifications. During the fourth week the capillaries are growing in first of all from the endosteum, though some vascular buds are visible in the periosteum as well, and at the same time the islet of cartilage is still very extensive. During the sixth week vascularisation is intense from both the endosteum and periosteum, and anastomoses are produced between the two networks. Histologically, the size of the islet of cartilage is reduced to a minimum. If we try to establish a parallellism between the two findings, we encounter difficulties right at the beginning. It is not known namely what value would represent the starting point and consequently it is not known whether the value obtained during the first week for respiration is an increased or a reduced one. It is a high value, as compared with the respiration of cartilage, and it is high also when we compare it with the value obtained by OLÁH and associates [20, 21] during the first week of regeneration of the articular surface. At any rate, the rather high rate of tissue respiration seems to be in harmony with the rather good vascularisation. But during the second week the tissue respiration of the external callus continues although there is a remarkable decrease in the ingrowth of blood vessels. In the nailed animals the external blood supply continues to deteriorate during the third week, the respiratory value decreases. During the fourth and the sixth weeks vascularisation increases, and yet oxygen consumption decreases further in the external callus of the nailed animals. During the first two weeks endosteal vascularisation is strongly reduced in the medullary nailed animals, which the external tries to remedy during the first week, with the respiration diminished, then during the third week an increase of respiration results for a while, followed by another decrease, in spite of the fact that from the third week on vascularisation starts gradually.

This comparison, too, reveals that respiration does not follow strictly parallelly vascularisation. Similar results have been obtained by OLÁH and associates [20, 21] in the case of the regenerating articular surface. Thus, in the seven day stage when vascularisation is most favourable, histologically cartilage-free, young granulation tissue is found, at the same time the rate of

tissue respiration is the lowest, as compared with the results obtained in later stages. And yet the development of the regenerating articular surface is accompanied by vascular pauperisation, by a continuous increase in the size of the islet of cartilage, but the oxygen consumption decreases. These changes, too, indicate that although there is a correlation between vascularisation and tissue respiration, there is no parallellism between the two.

2. There are data in the literature indicating that the enzymes involved in respiration do not exhaust their entire capacity, i.e. under certain conditions cell function can be maintained longer by increasing the capacity. So for example according to the papers by WARBURG [27, 28] the increase of OH ion concentration, or mechanical injury to the echinus eggs increases respiration. Similar evidence has been reported by other authors [1, 9, 24]. This way it is possible that in our experiments the damage caused by the decrease of vascularisation may be responsible for the initial increase in the rate of respiration, just as OLÁH and associates [20, 21] have assumed it to be the case with the regenerating articular surface. The adaptation studies of USHAKOV [26] yielded similar evidence. This author understands cellular adaptation to be an increase of resistance against noxae. According to his results, if not in the connective, supportive tissue, but in the muscle, too, there is an ability to strive to maintain the original situation. This the cells try to achieve through the function and metabolism of enzymes and proteins. Thus, obviously the supportive tissues, too, try to maintain normal conditions by significantly increasing metabolism, by mobilising existing reserves. It is likely that this increased, forced activity leads to a temporary overproduction. Though not exclusively, the decrease of vascularisation may, in our opinion, lead to damage to the cells and their metabolism. It is to be noted here that from among the enzyme systems the glycolytic one is rather active [17], but the succinate and citric acid dehydrogenase activities are not significant in cartilage [5]. From this also follows that the compensatory activity of the cells gets exhausted and in case the damage persists, the consequences of it become dominant in the cell, which will be manifest in a decrease of respiration.

3. When enumerating the factors that may be involved in the increase or decrease of respiration, the changes in the cell count of the granulation tissue, too, should be taken into consideration. It has namely been reported by WARBURG [27, 28] and NEUHAUS [18,19] that the increase in the cell count of a tissue goes together with an increase of cell respiration. This may, of course, possess significance, but in our case it is difficult to see how it may, because different types of cell appear in the different stages, as a result of which the changes in cell count can not be evaluated.

4. It may also be assumed that a metabolism of excitation arises, in response to certain, for example stressor, effects. Fracture of the bone may be considered in a sense to be an effect of this sort. This, too, may explain the

increased respiration observed initially, mainly in the external layers. Yet, we think that a stressor effect may not last two or three weeks, and that such an effect ought to exert the same influence both externally and internally.

On the basis of the above considerations, our results may be interpreted as follows.

In the animals with medullary nailing after the fracture the blood vessels are broken, the blood supply is impaired and as a result the tissues in the area of the external callus are damaged. To this the cells respond by hyperfunction, forced metabolism and respiration. Even the results obtained during the first week indicate that the damage developed immediately after fracture. This may be improved to some extent by the periosteal vascularisation, but this is not sufficient, and therefore oxygen consumption will be higher still during the second week. Later, however, the cells exhausted and injured by the deficiency of external and internal blood supply will no longer be able to meet the requirements of the changed conditions and respiration as well as metabolism will decrease. These changes in the conditions are indicated by the extensive islet of cartilage in the callus, the morphological structure of adaptation to the alteration in the conditions. The situation is similar in the external callus of the not nailed animals. The difference here is that the damage is not so extensive. The internal blood supply is not so severely affected, as a result of which the damage to the tissues develops at a lesser speed. This accounts for the fact that respiration reaches the maximum during the third week, is not so great in measure as in the nailed animals, and as a result of this the reduction ceases during the sixth week already. The situation in the case of the nailed internal callus is similar to that in the case of the external one, the only difference being that the maximum increment of respiration is found during the third week. The damage here exerts its effect slowlier, presumably because of the haematoma between the fracture ends, which may serve as a sort of depot for the internal callus. The severity of the damage is indicated by the fact that respiration is still diminished during the sixth week. We are unable to offer an acceptable explanation for the excessive increase of respiration in the internal callus of the not nailed animals during the third week.

In connexion with the results obtained the following should be noted. The facts that the fracture can compensate for two or three weeks following the decrease of blood supply by an increase of metabolism, or can overcompensate, at that, and that the nailed external callus is unable to compensate even at six weeks, although the blood supply was improved during the fourth week, indicate that in the chain of causes and effects a certain latency period is required. If we compare the development of callus in the nailed and not nailed animals from this respect, with regard to the respiratory values, one gets the impression that medullary nailing makes the damage caused by the fracture more severe, by causing a more extensive injury to the blood vessels. As a

result metabolism decreases to a greater extent and the morphological structure of adaptation, the cartilage cell, gains preponderance.

Finally, we should like to deal with the great range of variation of the results. It is known from literature that different species of dogs show differences in many respects, which may become manifest in callus formation as well. KROMPECHER [12 to 14], as well as OLÁH and associates [20, 21] mention this in connexion with the wilder and tamer animals, in the first place, as a factor influencing regeneration. This applies, of course, to our cases, too. For this reason we have tried to take into account such varietal differences with the grouping, to ensure identical conditions of maintenance, and to reduce the sources of error to a minimum by eliminating animals which refused to use their broken legs. Unfortunately, the respiration values obtained are relative ones. There is no absolute zero or any other standard value of reference. It is known from literature that the value of cartilage respiration is practically nil. Yet, as compared to that, we got very high values even in the severest stages. We wanted to compare the results with those obtained for intact bone or cartilage. But this way we cannot get the values for the respiration of bone, because the relation to dry solids in the case of bone, on account of its high calcium concentration, is a source of error which would make the results almost inevaluable. Thus, all we could do was to relate the values obtained to one another, which expresses what changes in respiration take place in the tissue of the callus at various stages of its development.

Acknowledgement

The authors express their thanks to Dr. B. GYIRES, Professor of the Institute of Applied Mathematics, Kossuth Lajos University of Sciences, for the calculations of significance.

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GEWEBSRESPIRATION WÄHREND REGENERATIVEN CHONDRALEN KNOCHENBILDUNG (KALLUSFORMATION)

Verfasser untersuchten mit der Warburgschen Technik die Gewebsatmung im äußeren und inneren Kallus von 74 Hunden. Bei einem Teil der Tiere wurden die Knochenfragmente mittels Knochenmarknagelung fixiert, bei der zweiten Gruppe erfolgte keine solche Fixierung. Die Untersuchungen wurden in der 1., 2., 3., 4., und 6. Woche nach der Fraktur vorgenommen. Bei den Hunden der ersten Gruppe zeigte der äußere Kallus anfangs einen Respirationsanstieg, der später, beginnend mit der dritten Woche, von einer Atmungsabnahme abgelöst wurde. Beim inneren Kallus ließ sich im großen und ganzen das gleiche beobachten. Im äußeren Kallus der nicht genagelten Hunde erreichte die Atmungssteigerung in der dritten Woche die maximalen Werte, in der vierten Woche ließ sich eine vorübergehende Abnahme registrieren, und in der sechsten Woche zeigte die Respiration wieder eine Steigerungstendenz. Im inneren Kallus konnte man in der dritten Woche außerordentlich hohe Respirationswerte messen, in der vierten Woche erfolgte aber eine starke Abnahme der Atmung, die im sechswöchigen Stadium wieder anstieg. Auf Grund der Besprechung der die Gewebsatmung beeinflussenden möglichen Faktoren gelangen die Verfasser zu der Folgerung, daß der Respirationsanstieg als Ergebnis des pathologischen Stoffwechsels infolge der nach der Fraktur auftretenden Vaskularisationsstörung entsteht, während das längere Fortbestehen und die Aufrechterhaltung der Schädigung zur Abnahme der Gewebsatmung führt. Die großen Knorpelzellen stellen die morphologische Manifestation dieser Atmungsabnahme dar. Parallel mit der Überhandnahme der Ossifikation können erneut höhere Atmungswerte gemessen werden. Auf Grund dieser Ausführungen scheint es, daß bei den Tieren nach der Küntscherschen Knochemarknagelung eine Schädigung größeren Ausmaßes vorliegt, und dementsprechend wird auch die Kallusbildung längere Zeit aufrechterhalten.

ДЫХАНИЕ ТКАНЕЙ ПРИ ВОССТАНОВИТЕЛЬНОЙ ОБРАЗОВАНИИ ХРЯЩЕВОЙ КОСТИ (ОБРАЗОВАНИЕ КАЛЛЮСА)

Авторы при помощи Варбургской техники исследовали условия тканевого дыхания внешней и внутренней мозолей 74 собаки. У одной части собак отломки соединили при помощи штифтов в костном мозге, у другой группы такая фиксация не применилась. Исследования проводились на 1., 2., 3., 4. и 6. неделе после перелома. У животных первой группы внешняя мозоль в начале показала повышение дыхания, а затем, начиная с третьей недели дыхание снизилось. Во внутренней мозоли в общем и целом наблюдалось подобное положение. Собаки второй группы, в которой не проводилось соединения отломков при помощи штифтов, во внешней мозоли повышение дыхания достигло максимальных величин на третьей неделе, на четвертой неделе оно временно снизилось, а на шестой неделе опять отмечалось тенденция к повышению дыхания. Во внутренней мозоли на третьей неделе отмечались весьма большие величины дыхания, на четвертой неделе респирация сильно снизилась, а затем, на шестой неделе она опять повысилась. На основании обсуждения возможных факторов, влияющих на тканевое дыхание, авторы приходят к тому заключению, что повышение дыхания создается в результате патологического обмена веществ, возникающего после перелома вследствие расстройства васкуляризации. Продолжительное существование или сохранение же повреждения вызывает снижение дыхания. Морфологическим признаком этого являются объемистые хрящевые клетки. Параллельно с продвижением окостенения опять наблюдаются более высокие величины дыхания. На основании сказанного кажется, что у животных после соединения отломков штифтами Кюнтшера повреждение более значительное и в результате этого хрящеобразование также более продолжительное.

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Acta Biol. Hung., **15** (1), 11—17 (1964)

DEVELOPMENT OF ACETYLCHOLINESTERASE-ACTIVE STRUCTURES IN THE RAT ARCHICEREBELLAR CORTEX

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(Received October 11, 1963)

Synopsis

Ontogenetical development of the acetylcholinesterase (AChE) active structures in the archicerebellar cortex of rats has been studied by means of histochemical methods. In the early phases of postnatal development the Purkinje cells gain a well-defined AChE activity, that decreases successively and disappears on the 20-th postnatal day. From the 7-th postnatal day on, AChE-active mossy fibres invade the white matter and the granular layer. It appears that the so called "critical period" in the development of cerebellum (Robins and Lowe) is associated with important alterations in the distribution of AChE in the archicerebellar cortex, that result in the adult pattern of this enzyme.

Introduction

According to the generally adopted view, in spite of its functional differentiation, all parts of the cerebellar cortex show a remarkable structural uniformity, both in morphological [2] and in histochemical [10] sense of the word. Recent investigations in this laboratory [3], however, revealed that the archicerebellar granular layer differs characteristically from that of the neocerebellar* areas, in that the former exhibits a markedly stronger acetylcholinesterase activity. It has been shown earlier that AChE activity in the granular layer is confined to the large presynaptic terminals of mossy fibres. Accordingly, the strong AChE activity of the archicerebellar granular layer is due to a high concentration of this enzyme in the mossy fibres.

The present paper deals with the ontogenetic development of these AChE-active archicerebellar structures, with special regard to the histochemical differentiation of mossy fibers and Purkinje cells.

Methods

Investigations were performed on cerebella of albino rats of various ages. In all, 8 embryos (ranging 14 to 21 days), 5 new-born rats, 24 baby rats (1 to 25 days) as well as 12 young and adult rats were used. The animals were sacrified by decapitation, the cerebella exposed and fixed in chilled 10% neutral formalin at 4° C for 4—6 hours. After fixation, paramedian frozen sections (20—25μ) were cut, rinsed in distilled water for 10 minutes and stained for AChE

* For the sake of simplicity, the term "neocerebellar" will be used to designate all the non-archicerebellar areas of the cerebellum, although it should be kept in mind that large parts of these areas are actually "paleocerebellar".

in the acetylthiocholine medium, according to the GEREBTZOFF modification [4] of the KOELLE technique [7]. Incubation has been carried out at pH = 5,8 for 80 minutes at 37° C. After incubation, the sections were rinsed in distilled water and developed in a 5% aqueous solution of sodium sulfide, rinsed again in distilled water and mounted in glue (gum arabic) without dehydration. — For differentiation between AChE and pseudocholinesterase parallel sections were pre-incubated for 30 minutes at room temperature in 4×10^{-8} M DFP or in 10^{-6}M Mipafox solutions.

The incubation parametra used in these investigations reveal the comparatively strong AChE activity of archicerebellar structures only, not staining the less active neocerebellar areas. It has been pointed out in previous studies that the AChE reaction in the archicerebellum is at least four times stronger than that in the neocerebellum. Extended incubation periods (e.g. 20 hours in SNELL's studies [9]) are able obscure the differences between the reactivities of archi- and neocerebellar areas, and what is more obscure also the real cytochemical localization of the enzyme. In our opinion, overincubation artifacts are responsible for the "AChE activity of granule cells" or that of stellate neurons, described by several investigators.

Results

In embryos, new-born rats as well as during the first two days of postnatal development, the cerebellar cortex does not show any sign of AChE activity, at least with the technique employed. Yet the nerve cells in the nuclei of the brain stem show clearly up as early as on the 18-th intrauterine day. The first cells exhibiting AChE activity in the cerebellar cortex appear at the 3rd postnatal day, close to the vestibular nucleus, right over the roof of the 4th ventricle. On the 4th day postnatally, a larger number of AChE active cells of the same size and shape appear in the area behind the posterolateral fissure. The enzyme is confined to granules in the cytoplasms of these cells, whereas the nucleus is devoid of AChE activity. (Cytochemical details of this process will be described in a second publication).

On the 5th postnatal day, similar cells appear in the nodule, located at the border-line between the molecular layer and the internal (definitive) granular layer (Fig. 1). The number of these AChE-active cells increases rapidly in the course of the following days, and on the 10th postnatal day AChE-active cells are crowded in the aforementioned localization throughout the nodule and the lower surface of the uvule (Fig. 2.). Until the 6th postnatal day, these cells possess an ovoid shape. From the 7th day on, however, apical dendrites exhibiting the same degree of AChE activity emerge from these cells, oriented towards the surface of the cortex (Fig. 3). Due to their histological features, these cells resemble the immature Purkinje cells described by Cajal ("state of dendritic disorientation") that lack the elaborate pattern of dendritic arborization, characterizing mature Purkinje cells (Figs. 4 and 5). It should be pointed out, furthermore, that although the great majority of Purkinje cells exhibited a strong enzymic activity, there could always be found inactive Purkinje cells intermingled with the active ones, essentially in the same histological localization, and of the same sizes and shapes as their AChE-positive neighbours.

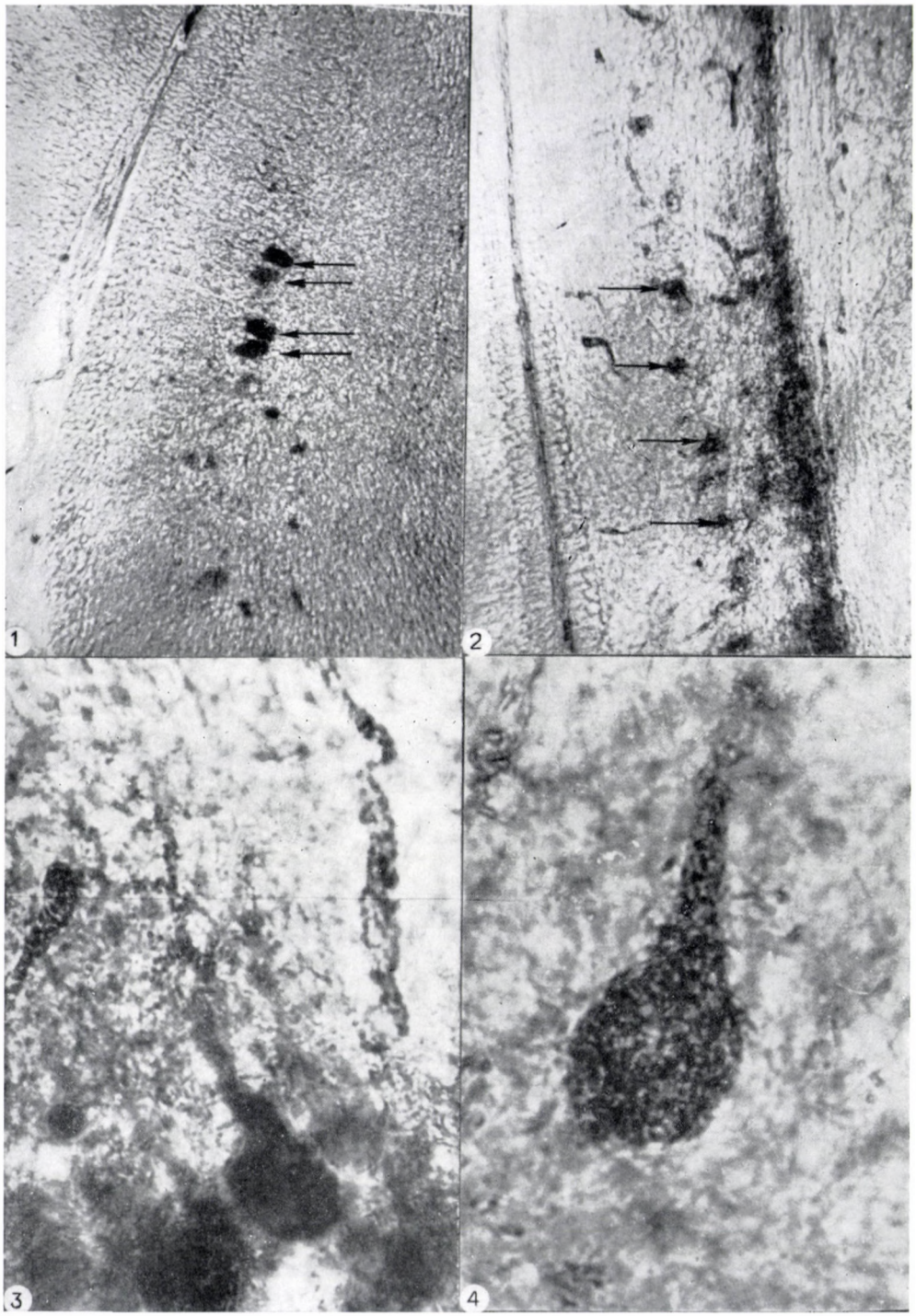

Fig. 1. Appearence of the first cells in the nodule (arrows) × 120 — *Fig. 2.* Numerical growth of the same cells in the following days. × 250 — *Fig. 3.* AChE-active cells and their enzyme active dendrite processes (arrow) × 750 — *Fig. 4.* Enzyme active Purkinje cell and its dendrite × 850

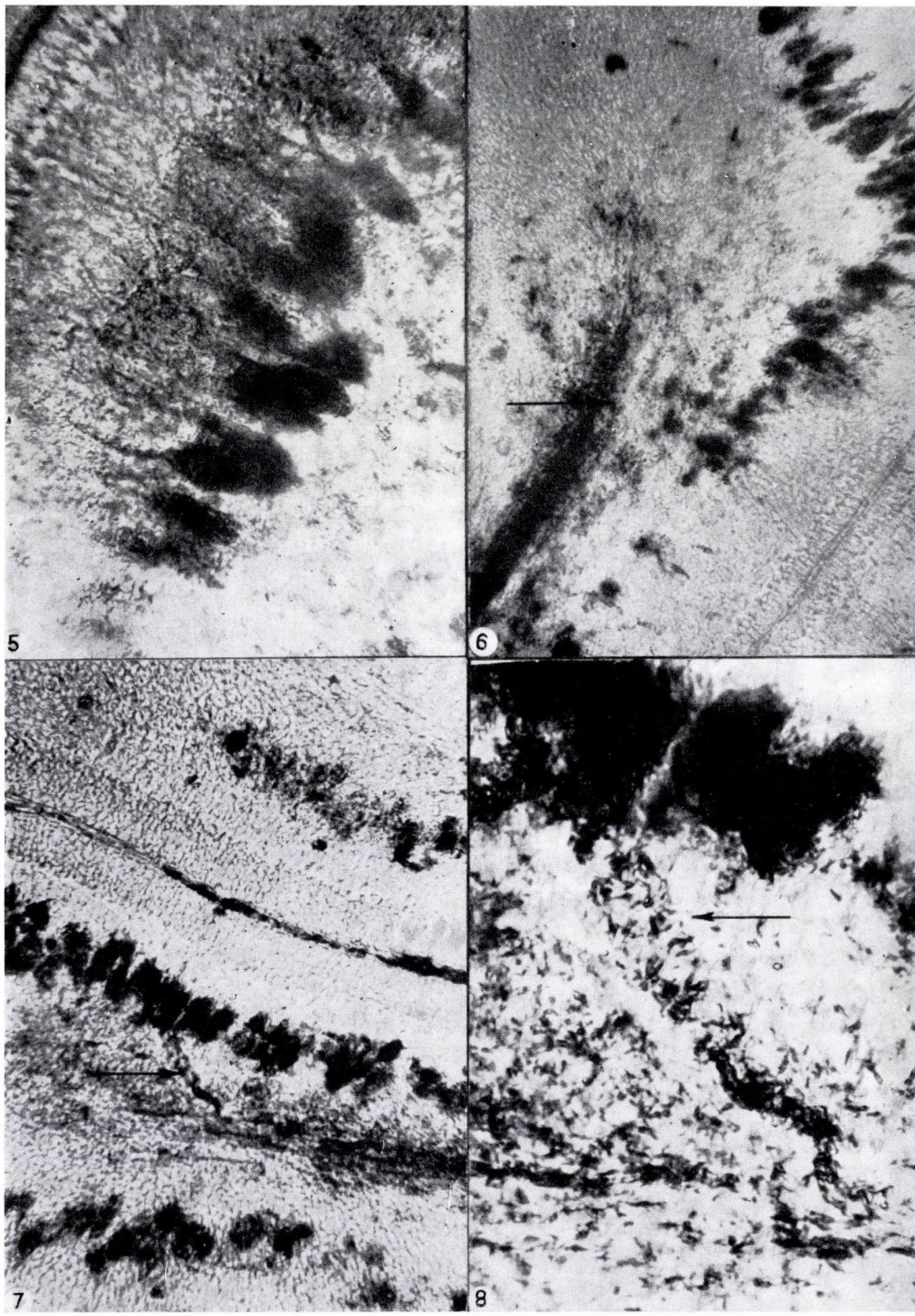

Fig. 5. Dendrites of enzyme active Purkinje cells in the "state of desorientation" × 550 — *Fig. 6.* Invasion of the mossy fibres into the archicerebellar nodule (arrow) × 150 — *Fig. 7.* Spiral structure of the mossy fibres (arrow) under the layer of the Purkinje cells in the area of the definitive granular layer. Low power. × 300 — *Fig. 8.* The same area, high power (arrow) × 1000

Shortly after the appearence of AChE-active Purkinje cells on the 7th postnatal day, nerve fibres exhibiting a similarly strong enzymic activity invade both the nodule and the lower uvula, i.e. the same territories where AChE-positive Purkinje cells had taken place already (Fig. 6). The endings of these AChE-active nerve fibers, located right beneath the row of Purkinje cells, are usually helicoidal, sometimes resembling the apparatuses of Perroncito (Figs. 7 and 8).

The conspicuously strong AChE-activity of the archicerebellar Purkinje cells persists until the 14th postnatal day. Beginning with the 15th day, however, their enzymic activity starts to decrease and disappears on the 20th postnatal day. The decrease of enzyme activity in Purkinje cells is temporarily correlated with the process of maturation that results in the definite pattern of their dendritic arborization. Activity of the mossy fibers and their end-apparatuses, however, remains unaltered both in this and in later periods of development.

Discussion

According to ADDISON's classical investigations [1], in the cerebellar cortex of the new-born rat four well-defined layers may be distinguished:

1. External (embryonal) granular layer
2. Molecular layer
3. Layer of Purkinje cells
4. Internal (definitive) granular layer.

In the course of later development, the external (embryonal) granular layer disappears, or, more properly, its nerve cells descend into the internal granular layer, whilst their processes remain in the superficial area, embodied later by the molecular layer. This process is virtually the same as that occurring during the development of the human cerebellar cortex, described originally by CAJAL [2] and later, in a more detailed form, by JAKOB [5]. In contrast to the rat, however, the disappearence of the external granular layer is completed in human embryos prenatally. Accordingly, the postnatal histochemical events observed in the rat cerebellar cortex can be regarded as the equivalent of the prenatal development in man.

The most striking histochemical phenomena in the rat cerebellar cortex are the appearence and disappearence of AChE-active mossy fibres in the same area. These developmental characteristics suggest that the archicerebellar region possesses marked cholinergic features as contrasted to other areas of the cerebellum. Although the reason of this strikingly strong AChE-activity of the archicerebellum is unknown, the explanation appears probable that it may be due to topographical and functional relations of this area to the vestibulocerebellar system, also characterized by a strong AChE-activity. In this

respect the archicerebellar area appears to be a separate part of the cerebellum, differing histochemically from structurally identical neocerebellar regions.

No definite explanation can be given for the conspicuous appearence and disappearence of archicerebellar Purkinje cells either. The fact that similar AChE-active cells could be primarily found in areas remote from the cerebellar cortex, might suggest that these cells immigrated into the cerebellum from brain stem sources, and settled down amongst other AChE-inactive Purkinje cells. On the other hand, the possibility cannot be excluded that some of the originally inactive Purkinje cells gain AChE activity during this postnatal period. As already mentioned, their activity decreases and disappears after a short blooming period. It appears, however, that the "disappearence" of the enzyme after the blooming period is to be regarded as a sign of a latent activity, since, under experimental conditions their AChE-activity reappears again, as shown by KÁSA and associates in our laboratory (unpublished).

The origin of the AChE-active mossy fibres invading the archicerebellar areas postnatally is also unknown at present. In serial sections of adult rat cerebella and brain stems they could be traced back to the vestibular nuclei that suggest that they represented secondary vestibulocerebellar fibres.

According to ROBINS and LOWE [8] "the rapid rate of protein and lipid deposition from 9 to 14 days, the profound histological changes during this period and the first appearence of adult electrical activity from the cerebellum at day 12, all suggest that the period of 9 to 14 days postnatally may correspond for the rat cerebellum the critical period". Our observations suggest that this critical period is associated with important histochemical events, too, resulting in the adult pattern of AChE-distribution in the archicerebellar cortex.

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ENTWICKLUNG VON STRUKTUREN MIT AZETYLCHOLINESTERASE-AKTIVITÄT IM ARCHICEREBELLUM VON RATTEN

Die ontogenetische Entwicklung von azetylcholinesterase-aktiven Strukturen in der archicerebellaren Kleinhirnrinde von Ratten wurde mit histochemischen Methoden untersucht. In der frühen Phase der postnatalen Entwicklung gewinnen die Purkinjezellen eine ausgeprägte Azetylcholinesterase-Aktivität, die stufenweise abnimmt und am 20. Tag nach der Geburt verschwindet. Beginnend mit dem 7. Tag nach der Geburt dringen azetylcholinesterase-aktiven Moosfasern in die weiße Substanz und in die Körnerschicht ein. Es scheint, daß die sogenannte »kritische Periode« in der Entwicklung des Kleinhirns (Robins und Lowe) mit wichtigen Änderungen in der Azetylcholinesterase-Verteilung im Archikortex des Kleinhirns einhergeht, und daß infolge dieser Änderungen der bei Erwachsenen beobachtete Verteilungstyp dieses Enzyms entsteht.

РАЗВИТИЕ СТРУКТУР С АЦЕТИЛХОЛИНЭСТЕРАЗНОЙ АКТИВНОСТЬЮ В ПЕРВИЧНОЙ КОРЕ МОЗЖЕЧКА У КРЫС

Авторы при помощи гистохимических методов исследовали онтогенетическое развитие структур с ацетилхолинэстеразной активностью в первичной коре мозжечка у крыс. В ранней фазе послеродового развития клетки Пуркинье приобретают хорошо выраженную ацетилхолинэстеразную активность, которая постепенно снижается и на 20-й день после рождения исчезает. Начиная с 7-го дня после рождения моховидные волокна с ацетилхолинэстеразной активностью проникают в белое вещество и зернистый слой. Кажется, что так называемый «критический период» развития мозжечка *(Робинс* и *Лоу)* связан с важными изменениями в распределении ацетилхолинэстеразы в первичной коре мозжечка, в результате чего получается взрослый тип распределения этого энзима.

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Acta Biol. Hung., **15** (1), 19—37 (1964)

STUDIES ON DISTRIBUTION OF N IN THE RAT DURING PREGNANCY

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(Received in revised form November 2, 1963)

Synopsis

Author has studied the quantity and distribution of "stored N" in the reproductive and vegetative organs of young rats (in the first reproduction cycle) and old ones (in the third reproduction cycle) during pregnancy. The N and water contents of the uterus, foetuses, lactiferous gland parenchyma, lactiferous gland teats (lactiferous glands), liver, gastrocnemius muscle, a predetermined area of abdominal skin, small intestine, femur, in the case of the animals in the third reproduction cycle also those of the heart, spleen and kidneys were determined. Amount of N retained during pregnancy by the maternal organism was determined indirectly, analysing the body constituents both of the experimental and control animals. The results indicate that two-thirds of the N retained by the organism during pregnancy are found in the reproductive organs and in the foetuses. The excess retention values for the 21-day pregnancy period are 1207 mg in the case of young animals, and 1575 mg N in that of the old ones.

From among the organs analysed, the uterus, lactiferous gland bodies, liver and gastrocnemius muscle of the rats in the first reproduction cycle contained N to a significantly lower percentile concentration than those of the non-pregnant controls.

Similar results have been obtained, except for the lactiferous gland bodies, in the case of old gravid rats.

In the reproductive and vegetative organs of the pregnant animals the absolute N content was increased only, as manifested by a significant surplus in the weight of the organs concerned.

Introduction

The maternal organism is known to retain significant quantities of N during pregnancy, as it can be measured and proved by different N balance metabolism studies.

Although the mean N retention values of certain species during gravidity are known, controversial evidence is found in literature as to the distribution of the retained N in the body. According to WHIPPLE's conception the most important, and probably exclusive, site of the incorporation and localisation of N would be the liver. There is much argument as to the validity of this view but no agreement of opinion has been reached as yet.

POO, LEW and ADDIS [17] found in pregnant rats a smaller increase of N content in the non-reproductive organs (those not involved directly in reproduction), while SPRAY and collaborators [19] have found that this increase was more considerable. At the same time BOYNE, CHALMERS and CUTHBERTSON [7] have found no increase of N content in the vegetative organs and tissues.

Studies by HÖFFSTRÖM [9] and HUNSCHER and collaborators [10, 11, 12] in pregnant women indicate that during pregnancy, and particularly in its advanced stages, the N content increases in the non-reproductive (vegetative) organs, although THOMPSON [20] has presented experimental evidence showing that it does not occur.

These examples make it clear that the opinions vary in the first place as to the quantitative distribution of the retained N between the reproductive and vegetative organs.

On the basis of direct N balance studies, POO and co-workers [17] have established that in the different organs of pregnant rats the measure of N storage varied with the changes in the protein content of food applied. PIKE [16] has arrived at the conclusion that the quantity of N retained depends on the calory intake, if sufficient protein is provided for. MORSE and SMITH [15] have reported that the N balance of adult rats varied from — 1 to + 2 g, if the N content of the foetuses was also taken into account.

BEATON and collaborators [1] called attention to the opposite changes in protein and fat metabolism in rats during gestation. They found namely, that until the 15th day of pregnancy fatty acids were accumulated in an excessive degree parallel to the water retention, whereas the retention of N was comparatively slightly increased. After the 15th day the opposite process took place: fat storage was excessively reduced and the retention of N increased. At present, there is no satisfactory explanation for this observation, although it seems to be doubtless that the significant retention of N during the second and final thirds of gestation is correlated with the hypertrophy and hyperplasia of the reproductive and vegetative organs, respectively. It has been demonstrated lately that the increase of N retention during the second half of pregnancy was not linear. According to MORRISON [14] during the last trimester the rate of increase of the N balance is slowing down, in some cases even negative values may appear, to be followed again by another more intense increase.

Although this analysis may prove that during pregnancy the protein synthesis in the organs and tissues increases, as suggested by the significant retention of N, it does not supply basis for making statements as to the relative distribution of the retained N between reproductive and vegetative organs. The authors quoted above obtained the numerical N retention values by two methods independent from each other, by direct N balance studies, on the one hand, and by analysing the body constituents of the experimental animals, on the other, thus determining the amount of N indirectly stored in the body during pregnancy.

In the present investigations we wished to determine the distribution of the N retained in the liver, reproductive organs, foetuses, vegetative organs and tissues of pregnant young rats (those in the first reproduction cycle) and of old ones (in the third reproduction cycle), and compare the results with

those obtained in non-pregnant animals. A secondary aim was also to assess the relationship between the changes in weight and water content of the organs showing alterations in the reproduction cycles, concerned.

Material and methods

A total of fifty albino rats of the ÉTI strain; 15 pregnant and 15 non-pregnant ones before the first reproduction cycle, as well as 10 pregnant and 10 non-pregnant ones before the third reproduction cycle were used.

The weight of the animals varied from 178 to 214 g, and 230 to 246 g, respectively.

The animals were fed ad libitum a 15 per cent protein standard diet. The animals were killed 24 to 36 hours before parturition, anaesthetised with ether and exsanguinated abdominally.

The liver was weighed immediately after excision, subsequently the lateral sinister lobe was analysed for total N by the Kjeldahl method, the medial, caudal and lateral dexter lobes were tested together for absolute dry matter content, after 3 hours of drying at 105° C.

Of the reproductive organs the uterus, the foetuses and other products of conception, the lactiferous gland parenchyma, the lactiferous gland teats (bodies) of the vegetative organs, the gastrocnemius muscle, duodenum, jejunum and ileum, the femur, a predetermined area of the abdominal skin, and in the case of animals in the third reproduction cycle the heart, spleen and kidneys were subjected also to study.

Moisture content was determined by drying at 105°C, N content by the Kjeldahl method, and fat content by the Soxleth extraction technique.

During dissection the femoral bone was freed mechanically from the muscle and tendon tissues adherent to it. The small intestine was invariably cut up longitudinally washed and blotted up with filter paper. The stomach, coecum and colon were treated in the same way, except that they were added to the body residue intended for homogenation or digestion.

The pregnant uterus was cut up, the foetuses were removed, weighed immediately and examined as outlined above.

The body residue (what remained of the body after the removal of the above mentioned reproductive and vegetative organs, and in the case of pregnant animals also that of the foetuses) was exposed to cold digestion with a determined quantity of concentrated sulphuric acid, accelerated by H_2O_2. The aliquot part pipetted out of the homogenous concentrate was tested for N content and thus the N value of body residue obtained.

By comparing the results of the body residue analysis with those obtained for the organs prepared out, we obtained the distribution of the numerical value of N throughout the body. The excess total body N over the control values gives the quantity of N retained during pregnancy. The following abbreviations, concepts are used in the text and tables.

Carcass (C): The body residue remained of the animal after removal of the organs specified above, and in the case of pregnant animals also of the foetuses and other conception products.

Organs dissected (Od): The isolated and examined organs and tissues together.

Carcass dissected (Cd): Body residue plus organs dissected.

Total carcass dissected (TCd): The carcass dissected of pregnant animal and the foetuses together (Cd+foetuses).

Results

We have studied in the young rats three kinds of N retention: one serving the general growth of the pregnant and non-pregnant animals alike, one providing for the synthesis of the foetuses and conception products of pregnant rats and thirdly, the super-retention of the amount of N partially covering the quantity of the milk proteins appearing during lactation.

Table 1 presents data indicating the increase of weight of the various organs during gestation. The greatest increase is found in the weight of the

Table 1

Changes in weight of organs examined of rats in the first

		1	2	3	4	5	6
		Uterus	Lactiferous parenchyma	Gland teat (gland)	Liver	Gastro-cnemius muscle	Abdominal skin
							Pregnant
First	$\bar{x}$	9.3796	6.2159	0.2920	9.3827	0.8523	0.1045
cycle	s	±1.7578	±1.3441	±0.0748	±0.9656	±0.1123	±0.0265
Third	$\bar{x}$	9.7759	7.6284	0.4555	11.6435	1.0589	0.1412
cycle	s	±1.2122	±1.0923	±0.0592	±1.3829	±0.1400	±0.0425
							Non-pregnant
First	$\bar{x}$	0.6926	1.2508	0.2470	7.1332	0.8143	0.0902
cycle	s	±0.2460	±0.3058	±0.0592	±0.8647	±0.1087	±0.0175
Third	$\bar{x}$	0.8527	1.5731	0.3475	7.8041	0.9625	0.1225
cycle	s	±0.2273	±0.4485	±0.0632	±1.3870	±0.1361	±0.0318

reproductive organs, so of the uterus (mainly of its myometrium) and in the lactiferous glandular tissues.

If the weight of organs, tissues of the non-pregnant rats are taken for 100 per cent, the increases in the weight of the uterus, lactiferous glandular parenchyma and lactiferous gland of rats in the first reproduction cycle are 1354, 497 and 118 per cent, respectively.

From among the vegetative organs, the pregnant rats' liver, gastrocnemius muscle (the two bundles of muscles together), abdominal skin area, small intestine and femoral bone weighed 32, 5, 16, 13 and 1.24 per cent was more than the corresponding organs of the non-pregnant controls of the same group.

In the pregnant rats the total of the organs prepared weighed 95 per cent more than in the controls.

The carcasses of the experimental rats weighed almost 5 per cent (in absolute value 7.957 g) more than those of the non-pregnant controls. This difference is an indicative of the general increase of body weight taking place during pregnancy.

As compared with the values obtained for the animals in the first reproductive cycle, those obtained for the rats in the third reproduction cycle were about 4 per cent higher in the case of the uterus, 23 per cent higher in that of the lactiferous glandular parenchyma and 6 per cent higher in the case of the lactiferous glandular bodies. The weight of the liver was 24 per cent, that of the gastrocnemius muscles together 24 per cent higher, the skin area's 35 per cent,

and third reproduction cycle (in grams)

7	8	9	10	11	12	13	14
Small intestine	Femur	Heart	Spleen	Kidney	Od	C	Foetuses
animals							
7.0580	1.1350	—	—	—	34.4200	175.8822	39.0207
±0.8544	±0.0928	—	—	—	—	±12.6668	±9.3372
8.5729	1.3321	1.1218	1.1071	2.2433	45.0806	221.3551	43.4016
±1.1265	±0.1591	±0.2306	±0.1915	±0.2000	—	±10.4380	±11.2290
animals							
6.2691	1.1211	—	—	—	17.6183	167.9251	—
±1.0113	±0.1158	—	—	—	—	±8.2040	—
7.1648	1.3630	0.9943	0.9676	2.1492	24.3013	202.7448	—
±0.8442	±0.1934	±0.1435	±0.1476	±0.1786	—	±15.4710	—

the small intestines' 21 per cent, the femoral bones' 17 per cent, the prepared organs' 31 per cent and the carcases, 26 per cent higher. The total weight of the foetuses was 11 per cent higher. In the case of the nonpregnant animals the uterus weighed 23 per cent, the lactiferous glandular parenchyma 26 per cent, the lactiferous glands 41 per cent more. The liver weighed 9 per cent, the gastrocnemius muscles 18 per cent, the skin area 36 per cent, the small intestines 14 per cent, the femoral bones 22 per cent more. The dissected organs weighed 38 per cent, the carcasses 21 per cent more than the corresponding ones of the young rats (those in the first reproduction cycle).

As compared with the controls, the increases in weight in the gravid animals were: uterus 1046 per cent, lactiferous glandular parenchmya 385 per cent, lactiferous glandular bodies 31 per cent, liver 49 per cent, gastrocnemius muscles 10 per cent, skin area 15 per cent, small intestine 20 per cent, prepared organs 86 per cent, carcasses 9 per cent.

The changes in the weight of the heart, spleen and kidneys were not significant in the tests of old animals.

The data indicate that in the rats in either of the two reproduction cycles the liver and the small intestine showed the greatest increase of weight among the vegetative organs, beside the excessive increase in the weight of the organs directly involved in reproduction. (In the first experiment: $P\% < 0.10$ and $P\% < 2.90$, in the second experiment $P < 0.10$ and $P < 0.45$.)

In Table 2 are shown the percentage N content values of the individual reproductive and vegetative organs.

Table 2

Percentile N content of organs analyzed of rats in the first

		1	2	3	4	5	6
		Uterus	Lacti-ferous parenchyma	Gland teat (gland)	Liver	Gastrocn-emius muscle	Abdominal skin
							Pregnant
First	$\bar{x}$	1.90	2.23	3.85	3.06	2.89	4.94
cycle	s	±0.1338	±0.1905	±0.1803	±0.1179	±0.1073	±0.1169
Third	$\bar{x}$	1.88	2.29	3.92	2.97	2.84	5.02
cycle	s	±0.1113	±0.1625	±0.2119	±0.1744	±0.1127	±0.2218
							Non-pregnant
First	$\bar{x}$	2.29	2.26	4,29	3.18	3.01	5.00
cycle	s	±0.2343	±0.1516	±0.2018	±0.0969	±0.1504	±0.0980
Third	$\bar{x}$	2.12	2.31	3.98	3.15	3.04	5.18
cycle	s	±0.2938	±0.1183	±0.2965	±0.1876	±0.1658	±0.2917

It appears in both the first and third reproduction cycle the reproductive and vegetative organs of the pregnant rats contain less percentile N than those of the non-pregnant control animals. The differences were in the first experiment: uterus 0.39 g per cent ($P\% < 0.10$), lactiferous glandular parenchyma 0.03 g per cent ($P\% < 62.0$), lactiferous glands 0.44 g per cent ($P\% < 0.10$), liver 0.12 ($P\% < 0.55$), gastrocnemius muscles 0.12 g per cent ($P\% < 0.70$), skin area 0.06 g per cent ($P\% < 14.3$), small intestine 0.04 g per cent ($P\% < 42.8$), femurs 0.03 g per cent ($P\% < 76.5$).

As the values of P indicate, the differences were significant in the case of the uterus, lactiferous glands, liver and muscles only. The lactiferous glandular parenchyma contained approximately a constant concentration of N during gestation. However, it is to be emphasized that the absolute N content of the parenchymal tissue was much higher than that of the controls, because the lactiferous glandular parenchyma increases to fivefold and fourfold the original weight during gestation, respectively, and therefore it is one of the most important N-storing tissues among the extrahepatic protein depots.

The difference in the N g per cent values was not significant in the case of skin, small intestine and femur.

Likewise, there was no significant difference between the pregnant and non-pregnant rats in respect of the N content related to absolute dry matter content. The gram per cent N values were: uterus 9.78 (9.86), lactiferous glands 12.35 (11.59), liver 10.34 (10.35), gastrocnemius muscles 12.87 (12.71), skin 13.34 (14.60), small intestine 9.16 (9.25), femur 4.32 (4.21) g per cent, respectively. (In parenthesis the values obtained in the controls are shown.)

and third reproduction cycle (in grams)

7	8	9	10	11	12	13
Small intestine	Femur	Heart	Spleen	Kidney	C	Foetuses
animals						
1.92	3.31	—	—	—	2.73	1.55
±13,38	±0,2359	—	—	—	±0.1556	±0.2035
2.06	3.18	2.80	3.49	2.97	3.06	1.49
±0,0866	±0,1349	±0.2422	±0.3927	±0.0837	±0.1175	±0.1827
animals						
1.96	3.34	—	—	—	2.72	—
±0,1417	±0,2023	—	—	—	±0.1253	—
2.07	3.27	2.84	3.55	3.00	3.10	—
±0,1507	±0.2010	±0.2870	±0.2181	±0.1902	±0.1094	—

The results obtained for the animals in the third reproduction cycle differ only slightly from those obtained in the group of animals in the first reproduction cycle. The percentile differences were: uterus 0.24 g per cent ($P\% < 2.6$), lactiferous glandular parenchyma 0.02 g per cent ($P\% < 76.6$), liver 0.18 g per cent ($P\% < 4.0$), gastrocnemius muscle 0.20 g per cent ($P\% < 0.45$), abdominal skin 0.16 g per cent ($P\% < 17.7$), small intestine 0.01 g per cent ($P\% < 84.1$), femur 0.09 g per cent ($P\% < 24.5$), heart 0.04 g per cent ($P\% < 76.6$), spleen 0.06 g per cent ($P\% < 69.2$), kidneys 0.03 per cent ($P\% < 62.2$). There were only slight differences in the g per cent N values between the lactiferous glands of the pregnant and nonpregnant animals (0.06 g per cent).

In the second experiment the P values of the uterus and liver show a smaller percentile difference, which may be ascribed first of all to the smaller anabolic activity of the older animals. In the vegetative organs, in which protein production is not marked, the "t" value of the percentile difference was low, and therefore the data were not significant.

The percentage differences between the carcass values were 0.01 per cent ($P\% < 84.1$) in the first reproduction cycle and 0.04 g per cent ($P\% < 43.1$) in the case of older animals.

The foetuses contained 1.55 and 1.49 g per cent N, respectively.

Considerable differences in absolute N content were found first of all in the case of the reproductive organs, whilst the liver and small intestine contained less, but significant amounts of N.

The absolute N content values for the organs are shown in Table 3. It appears that the increased N content of the organs, despite the lower percent-

Table 3

Absolute N content of organs examined of rats in the first

		1	2	3	4	5	6
		Uterus	Lacti-ferous parenchyma	Gland teat (gland)	Liver	Gastroc-nemius muscle	Abdomi-nal skin
							Pregnant
First	$\bar{x}$	0.1780	0.1366	0.0112	0.2868	0.0246	0.0052
cycle	s	±0.0345	±0.0231	±0.0084	±0.0941	±0.0038	±0.0084
Third	$\bar{x}$	0.1833	0.1756	0.0178	0.3444	0.0301	0.0072
cycle	s	±0.0236	±0.0300	±0.0033	±0.0392	±0.0047	±0.0033
							Non-pregnant
First	$\bar{x}$	0.0159	0.0283	0.0106	0.2271	0.0245	0.0045
cycle	s	±0.0060	±0.0076	±0.0084	±0.0945	±0.0038	±0.0084
Third	$\bar{x}$	0.0180	0.0366	0.0138	0.2455	0.0291	0.0063
cycle	s	±0.0058	±0.0120	±0.0033	±0.0426	±0.0033	±0.0238

Table 4

Distribution of the surplus N retention values of rats in the first and third reproduction

First reproduction cycle				
Organs	Pregnant animals	Non-pregnant animals	Absolute surplus in g	Percentile distribution
Uterus	0.1780	0.0159	0.1621	13.428
Lactiferous gland parenchyma	0.1366	0.0283	0.1083	8.972
Lactiferous gland	0.0112	0.0106	0.0006	0.050
Liver	0.2868	0.2271	0.0597	4.946
Gastrocnemius muscle	0.0246	0.0245	0.0001	0.008
Abdominal skin	0.0052	0.0045	0.0007	0.058
Small intestine	0.1350	0.1226	0.0124	1.027
Femur	0.0376	0.0374	0.0002	0.018
Foetuses	0.6350	—	0.6350	52.605
C	4.8003	4.5723	0.2280	18.888
Total	—	—	1.2071	100.000

and third reproduction cycles (in grams)

7	8	9	10	11	12	13	14
Small intestine	Femur	Heart	Spleen	Kidney	Od	C	Foetuses
a n i m a l s							
0.1350	0.0376	—	—	—	0.8150	4.8003	0.6350
±0.0139	±0.0038	—	—	—	—	±0.3638	±0.1258
0.1757	0.0422	0.0314	0.0386	0.0666	1.1129	6.7689	0.6567
±0.0231	±0.0058	±0.0067	±0.0075	±0.0075	—	±0.4053	±0.4264
a n i m a l s							
0.1226	0.0374	—	—	—	0.4738	4.5723	—
±0.0181	±0.0038	—	—	—	±0.4709	±0.9916	—
0.1488	0.0444	0.0282	0.0343	0.0643	0.6693	6.2945	—
±0.0058	±0.0047	±0.0047	±0.0047	±0.0033	—	±0.4309	—

cycle, on the basis of the absolute differences measured in the organs concerned

Third reproduction cycle				
Organs	Pregnant animals	Non pregnant animals	Absolute surplus in g	Percentile distribution
Uterus	0.1833	0.0180	0.1653	10.497
Lactiferous gland parenchyma	0.1756	0.0366	0.1390	8.827
Lactiferous gland	0.0178	0.0138	0.0040	0.254
Liver	0.3444	0.2455	0.0989	6.281
Gastrocnemius muscle	0.0301	0.0291	0.0010	0.064
Abdominal skin	0.0072	0.0063	0.0009	0.058
Small intestine	0.1757	0.1488	0.0269	1.708
Femur	0.0422	0.0444	—0.0022	—0.140
Heart	0.0314	0.0282	0.0032	0.203
Spleen	0.0386	0.0343	0.0043	0.273
Kidney	0.0666	0.0643	0.0023	0.146
Foetuses	0.6567	—	0.6567	41.703
C	6.7689	6.2945	0.4744	30.126
Total	—	—	1.5747	100.000

age values, originates from the hypertrophic changes. By adding up the differences in the absolute N contents of the dissected organs, foetuses and body residues we obtained the total N retention value of the pregnant organism; thus, in the first reproduction cycle the total N retention amounts to 1207 mg, in the third reproduction cycle to 1575 mg. When taking the total retention values for 100 per cent, in the case of the young animals the following percentage N retention values are obtained: carcass 19 per cent, uterus 13 per cent, lactiferous glandular parenchyma 9 per cent, liver 5 per cent, small intestine 1 per cent, lactiferous glands 0.05 per cent and foetuses 53 per cent. In the old animals the N retention values are: carcass 30 per cent, uterus 10 per cent, lactiferous glandular parenchyma 9 per cent, liver 6 per cent, foetuses 42 per cent (see Table 4).

In Table 5 the percentage and absolute N content values for the body residue, the dissected and foetus-bearing bodies are presented. In both the first and third reproduction cycle, in the pregnant and non-pregnant animals, the body carcasses contain similar percentages of N. In the case of the dissected body the difference is marked, due to the higher moisture content of the pregnant organism (see later). The most marked difference is found in the case of the percentile N content of the foetus-bearing bodies.

Table 5

Absolute and percentile N contents of carcasses (C) and carcasses dissected (Cd) of rate in the first and third reproduction cycle in grams

	First reproduction cycle			
	Group of animals	C	Cd	TCd
Absolute	Nonpregnant	4.5723	5.0432	—
	Pregnant	4.8003	5.6153	6.2503
Percentile	Nonpregnant	2.72%	2.72%	—
	Pregnant	2.73%	2.67%	2.51%

	Third reproduction cycle			
	Group of animals	C	Cd	TCd
Absolute	Nonpregnant	6.2945	6.9638	—
	Pregnant	6.7689	7.8818	8.5385
Percentile	Nonpregnant	3.10%	3.07%	—
	Pregnant	3.06%	2.96%	2.76%

Table 6

Distribution of the N amounts "stored" in the carcasses concerned of rats in the first and third reproduction cycle

First reproduction cycle Pregnant animals		Percentile distribution
"C" contains g N	4.8003	85.4861
"Od" contain g N	0.8150	14.5139
"Cd" contain g N	5.6153	100.0000

First reproduction cycle Non pregnant animals		Percentile distribution
"C" contains g N	4.5723	90.6627
"Od" contain g N	0.4709	9.3373
"Cd" contains g N	5.0432	100.0000

Third reproduction cycle Pregnant animals		Percentile distribution
"C" contains g N	6.7689	85.8801
"Od" contain g N	1.1129	14.1199
"Cd" contain g N	7.8818	100.0000

Third reproduction cycle Non pregnant animals		Percentile distribution
"C" contains g N	6.2945	90.3889
"Od" contain g N	0.6693	9.6111
"Cd" contains g N	6.9638	100.0000

Table 7

Percentile and absolute moisture content of the organ

		1	2	3	4
		Uterus	Lactiferous parenchyma	Gland teat (gland)	Liver
					Pregnant
Percentile values	$\bar{x}$	80.58	64.32	68.82	70.42
	s	±1.8423	±4.3680	±2.9100	±2.7385
Absolute values	$\bar{x}$	7.5633	3.9724	0.2011	6.6130
	s	±1.4687	±0.7716	±0.0541	±0.8702
					Non-pregnant
Percentile values	$\bar{x}$	76.78	59.85	62.98	69.57
	s	±2.9193	±5.6650	±2.3276	±1.6696
Absolute values	$\bar{x}$	0.5337	0.7436	0.1553	4.9651
	s	±1.1967	±0.1729	±0.0374	±0.6239

The values of the absolute N quantities found in the carcasses concerned, and in the case of pregnant animals in the total carcasses dissected prove the absolute N "storage" and/or retention only.

If we examine the absolute N content of the carcasses in the individual reproduction cycles, distributed between the carcass and the organs dissected, we shall note that in the first experiment 14.51 per cent of the total N of the carcass tested, in the second experiment 14.12 per cent, in the nonpregnant rats 9.34 and 9.61 per cent respectively, related to the organs analysed.

Accordingly, in the pregnant animals the 85.49 and 85.88 per cent, and in the controls the 90.66 per cent and 90.39 per cent values relate to the absolute N content of the body residues, respectively (Table 6).

The fact that in the pregnant rats a much higher proportion of the absolute N content of the carcass dissected falls to the absolute N content of the organs dissected, than in the controls, indicates that the organs examined are definitely capable of "storing" N.

The absolute and per cent water content values for the organs of rats in the first reproduction cycle are presented in Table 7.

It appears from the table that the reproductive organs contain much more moisture, than the corresponding organs of the controls.

The per cent differences are: uterus 3.80 g per cent ($P\% < 0.10$) lactiferous glandular parenchyma 4.47 g per cent ($P\% < 2.3$), lactiferous glands 5.84 g per cent ($P\% < 0.10$). These proved to be significant. The differences found in the case of the liver (0.85 g per cent, $P\% < 32.4$), gastrocnemius muscle (1.46 g per cent, $P\% < 8.1$), abdominal skin (1.04 g per cent, $P\% < 48.7$),

examined of rats in the first reproduction cycle

5	6	7	8	9	10
Gastrocnemius muscle	Abdominal skin	Small intestine	Femur	Foetuses	Od
animals					
77.55	65.56	79.04	23.38	85.68	—
±2.8871	±4.2105	±4.2500	±3.8110	±3.6000	—
0.6622	0.0683	5.5857	0.2650	53.2229	24.9310
±0.1029	±0.0173	±0.7824	±0.0480	±6.9900	—
animals					
76.09	64.52	77.62	22.37	—	—
±0.8231	±3.7780	±32.015	±3.8830	—	—
0.6195	0.0578	4.8636	0.2531	—	12.1917
±0.0819	±0.0037	±0.8049	±0.0592	—	—

small intestine (1.42 g per cent, $P\% < 32.4$) and femur (1.01 g per cent, $P\% < 48.7$) were not significant. The average foetal moisture content was 85.68 ± 3.60 g per cent. As in the case of the percentile and absolute N content values of the organs, the standard deviations show over numerical values similarly, in the case of the absolute water content, than in the case of the percentile moisture content. This follows from the relatively low s.d. values of the organs concerned.

As regards the absolute moisture content of the organs examined, distribution of the absolute difference between the dissected organ values of pregnant and non-pregnant animals (12.7393 g = 100 per cent) is as follows: the higher water content measured in the pregnant animals relates from among the organs examined in 55.18 per cent to the uterus, in 25.35 per cent to the lactiferous glandular parenchyma and in 0.36 per cent to the lactiferous glands. From the remainder proportion of the liver is 12.94 per cent, of the gastrocnemius muscle 0.34 per cent, of the abdominal skin 0.08 per cent, of the small intestine 5.67 per cent and of the femur 0.08 per cent.

In the first reproduction cycle the dissected rat bodies contained an average of 69.57 g per cent, in the third 68.93 g per cent of moisture, as compared with the 65.77 and 65.57 g per cent values obtained for the controls, as related to the original weight. The absolute moisture content values were 144.80 g (first reproduction cycle), 141.49 g (third reproduction cycle), and 120.71 g and/or 120.57 g (controls). The difference is significant in both cases ($P\% < 2.9$ and $P\% < 0.18$). The deviations were greater in the case of the younger animals.

The per cent values for fat-free weight were: 78.41 g (first reproduction cycle), 78.46 g (third reproduction cycle), and 74.23 and/or 74.47 g per cent (controls).

The results prove that during pregnancy hydraemia ensues.

Table 8

Percentile and absolute body-fat content of rats in the first and third reproduction cycle in grams

		First reproduction cycle		Third reproduction cycle	
		pregnant	non-pregnant	pregnant	non-pregnant
		animals			
Percentile values	$\bar{x}$	8.84	8.46	9.53	8.90
	s	±1.6202	±1.8265	±1.9911	±1.3758
Absolute values	$\bar{x}$	18.2972	15.7633	19.7040	16.8820
	s	±3.1827	±3.0443	±4.7276	±3.2227

Table 8 presents the data for the percentile and absolute fat contents of the total carcasses dissected. The values found were: 8.84 g per cent (pregnant rats of the first reproduction cycle), 9.35 g per cent (third reproduction cycle), as compared with the control values of 8.46 g per cent and 8.90 g per cent, respectively.

The absolute values were: young rats 18.30 and 15.76 g, old ones 19.70 and 16.88 g, respectively.

The differences between the experimental groups (first reproduction cycle: 0.38 g per cent, third reproduction cycle 0.63 g per cent) were not significant in the case of either the young animals ($P\% < 55.4$) or the old ones ($P\% < 33.0$).

Discussion

The results described make it clear that during gestation the organism "stores" the quantity of protein, ensuring the physiological conditions of reproduction exclusively through tissue hypertrophy and hyperplasia. The intensity of the N anabolism of the body depends on many factors. One of the most important one is the placenta. We should like to point out that in the changes of body weights the stimulating effect of the placenta is the primary factor. The investigations of BOURDEL and JACQUOT [4, 5, 6] have revealed that the placenta plays a significant role not only in this, but also in the regulation of N anabolism. These authors have shown that the most important part played by the placenta is to maintain N balance during gestation, even when nutrition is deficient. In rats, from which the ovaries and foetuses were removed

by operation, an average of 91 mg N was retained between the 15th and 20th days of pregnancy. In the same period the animals whose placenta was removed, retained only 4 mg N. The investigations of BOURDEL and JACQUOT indicate that if the feeding is deficient, the retained N is transferred to the foetuses, in the first place and the hyperplasia of the liver and the increase of the lactiferous glandular parenchyma will be reduced. If the foetuses are removed, the liver will show the physiological weight increase similarly of 30 to 40 per cent already referred to above.

The total N retention values obtained (1207 mg in the first experiment, and 1575 mg in the second experiment) are similar to those published by BOYNE, POO, ROMBAUTS [7, 17, 18]. BOYNE and coworkers have found the N retention related to the nonpregnant animals of the control group to amount to 1666 mg in pregnant rats 90 to 110 days old. In young animals also, ROMBAUTS has measured 630 mg N retention, in which, however, the N contents of the uterus and foetuses are not included. POO examined 100-day old rats and found the excess N retention to amount to 671 mg, as determined by analysing the single organs. It is to be noted that in the last trimester of pregnancy the direction tangent of the N retention curve cannot be considered as a constant. We refer to the results published by BEATON and MORRISON [1, 14]. who found that from the 16th day on N retention may decrease periodically. However, this is in an apparent contradiction with the observation that the storage of fat observed in the first half of gestation is followed by an increased storage of protein, we must say that in the last stage the anabolism of the reproductive organs continues to be high, though the amounts of protein accumulated by the vegetative tissues, decrease. It should be noted that in this last trimester the synthesis of the foetuses and other conception products can be represented invariably by an exponential curve.

The reduction of the per cent N content of the organs is explained by the high moisture content, in the first place. Many workers have reported that during pregnancy the N content of the vegetative organs increases [9, 10, 11, 12, 17, 19]. As contrast BOYNE and KENNEDY [7, 13] trace back the retention values of pregnant organisms to the increase in the size of the liver, exclusively reproductive organs and to the synthesis of conception products.

The increase moisture content of the reproductive organs and foetuses is explained by the high rate of cell multiplication, by the higher moisture content accompanying protein synthesis.

The hydraemic changes of vegetative organs, and their causes have already been referred to, but it is to be noted that the evidence in the literature concerning the changes in the water content of the liver during pregnancy is rather controversial. We quote BOKELMANN and SCHERINGER [2, 3], in the first place, who have found that beside the increase in the weight of the liver, as related to the total body weight, its dry matter content is just as constant

as its per cent N content. BEATON and coworkers [1] have found that the moisture content of the liver was increased in the pregnant rat. Thus, the difference between the livers of pregnant and non pregnant rats on the 8th day of gestation was 0.3 g per cent, on the 12th day, 0.5 g per cent, on the 14th day, 0.1 per cent, on the 16th ("metabolic turning point"), 1.3 g per cent ($P\% < 2.0$), on the 18th, 1.3 g per cent ($P\% < 1.0$) and on the 20th 0.7 g per cent ($P\% < 5.0$). The deviations showed a much smaller range than in our studies. BEATON and coworkers note that on the 5th day of pregnancy the carcasses of the eviscerated animals contained an average of 101.20 g of water, as compared with the 102.00 g obtained for the carcasses of the non-pregnant animals.

The water uptake values were: 8th day of pregnancy 6.2 g, 12th day 7.5 g, 14th day 11.8 g, 16th day 7.0 g, 18th day 13.8 g, 20th day 11.9 g and immediately before parturition 3.4 g, as compared with the control values of 0.5, 2.5, 0.5, 0.2, 0.7, 3.1 and 0.2 g.

The results outlined above show that water retention increases with the advance of pregnancy. As we have already mentioned, this is explained in the first place by the hight moisture content of the conception products synthesized at a very fast rate. Although it cannot be doubted that with the advance of pregnancy the various haemodynamical factors and the chemism of tissues continue to gain significance, nevertheless the protein synthesis that determines decisively the magnitude of the hydraemic conditions.

The opinions relative to the increase in the water content of the liver are not uniform. Beside the tissue chemism, possible changes in the moisture content of the liver may be caused by the increase in protein anabolism.

The studies of the changes in the water content of tissues are closely correlated with those concerned with the changes in the neutral fat content of the body. BEATON and collaborators [1] have reported that in the first period of pregnancy the tissues take up increased amounts of fats. As we have mentioned, this is inversely proportional to the ever increasing positive N balance. In Table 9 we present the data published by BEATON and collaborators to numerically corroborate those outlined above.

The data in Table 9 indicate that unlike fat uptake, it is only after the 16th—18th days that protein is localised, and also that in the second half of pregnancy the intensive synthesis of conception products demands for itself the materials retained by the maternal organism. As a result, in the final stage of pregnancy the fat and moisture, and also the protein contents of the vegetative body (maternal body deprived of the foetuses) do decrease.

POO and coworkers [17] report that during pregnancy the fat content of the different organs increases, with the exception of the alimentary tract (whose absolute protein content increases by 25 per cent during pregnancy).

If we realize that the excess protein contributed to the "depot proteins" determined in the course of the previous N balance studies does not suffice at

Table 9

Changes in the components of decapitated and eviscerated rat carcasses ("vegetative carcasses")

Days of pregnancy	Pregnant		Moisture content g	Nonpregnant		Moisture content g
	Fat g	Protein g		Fat g	Protein g	
Initial values	19.8	37.6	101.2	17.8	40.5	102.0
8th	+2.1	+1.7	+ 6.2	+1.9	—2.4	—0.5
12th	+4.5	+2.4	+ 7.5	+3.3	+0.4	+2.5
14th	+6.5	+3.2	+11.8	+3.5	+2.7	+0.5
16th	+5.7	+2.4	+ 7.0	+1.4	+1.0	—0.2
18th	+1.5	+7.0	+13.8	+3.7	—1.4	+0.7
20th	+0.4	+4.9	+11.9	+1.5	—0.8	+3.1
22nd	—0.5	+0.1	+ 3.4	—0.3	—2.2	—0.2

all to cover the need of milk protein production, we must seek the explanation in the changes of absorption taking place later in pregnancy and during lactation. One of the resultants of these may be a difference, manifested also in the changes observed, in different parts of the alimentary tract (in the first experiment in the case of the small intestine $P\% < 2.9$, in the second experiment $P\% < 0.45$). The above analysis is in part supported by the investigations, according to which also during lactation the size expressed in per cent of the body weight (absorption surface) and weight, first of all of the small intestine, to a lesser extent those of the stomach, coecum and large intestine, surpass significantly those of the non-lactating, control rats. (Personal communication B. F. FELL and R. M. CAMPBELL [1962], and investigations of PÉNZES [1963].)

The characteristic features of the changes in fat metabolism during pregnancy should be studied not in the viscera, in the carcass deprived of the reproductive organs only, but in the blood plasma, in the first place. Although the increase of ketone bodies in serum indicates clearly the changes in lipid metabolism (expressed in beta-oxybutric acid value, this corresponds to an average of 30.43 per cent), but the characteristic values appear in the quantities of neutral fat, fatty acids, total and free cholesterol and phospholipid. According to EFFKEMANN [8] the increases are: neutral fat 136.91 mg per cent, fatty acid 115.28 mg per cent, total cholesterol 11.17 mg per cent, free cholesterol 18.52 mg per cent and phospholipid 30.53 mg per cent.

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TICKSTOFFLOKALISATION WÄHREND DER GRAVIDITÄT IM ORGANISMUS VON RATTEN

Verfasser untersuchte in den reproduktiven und vegetativen Organen von jungen (im ersten Reproduktionszyklus befindlichen) und alten (in dritten Reproduktionszyklus befindlichen) Ratten die Menge des während der Gravidität im Organismus »gespeicherten« Stickstoffs sowie dessen Verteilung. Die Untersuchungen erstreckten sich auf die Analyse des Stickstoff- und Wassergehaltes der Gebärmutter, der Föten, des Parenchymgewebes der Milchdrüsen, der Milchdrüsenknospen (Milchdrüsenkörper) bzw. der Leber, des M. gastrocnemius, eines bestimmten teils der Bauchhaut, des Dünndarms sowie des Schenkelbeines (im Falle von Tieren im dritten Reproduktionszyklus — des Herzens, der Milz und der Nieren).

Die während der Gravidität durch den Mutterorganismus retinierte Stickstoffmenge wurde während der Analyse der Körperzusammensetzung der Versuchs- und Kontrolltiere, d.h. auf indirekten Wege festgestellt.

Aus den Ergebnissen ist ersichtlich, daß zwei Drittel der während der Gravidität im Organismus retinierten Stickstoffmenge in den reproduktiven Organen bzw. in den Föten gemessen wurde. Der Retentions-Mehrwert der 21-tägigen Graviditätsperiode beträgt im Falle von jungen Tieren 1207 mg, bei alten Tieren 1575 mg Stickstoff.

In den untersuchten Organen war der prozentuale Stickstoffgehalt der Gebärmutter, der Milchdrüsenknospen (Milchdrüsenkörper), der Leber und des M. gastrocnemius von Ratten des ersten Reproduktionszyklus signifikant geringer, als bei nicht graviden Kontrolltieren.

ЛОКАЛИЗАЦИЯ АЗОТА В ОРГАНИЗМЕ КРЫС ВО ВРЕМЯ БЕРЕМЕННОСТИ

Автор исследовал в воспроизводительных и вегетативных органах молодых (находящихся в первом воспроизводительном цикле) и старых (находящихся в третьем воспроизводительном цикле) крыс количество азота, «накопленного» в организме во время беременности, а также его распределение. Проводился и анализ содержания азота и воды в матке, плоде, паренхиматозной ткани молочных желез, сосках молочной железы, печени, икроножной мышце, определенной части кожи живота, тонкой кишке, а также бедренных костях, в случае животных третьего воспроизводительного цикла — в сердце, селезенке и почках. Автор определил количество азота, задержанного во время беременности материнским организмом в ходе анализа состава организма подопытных и контрольных животных, то есть косвенным путем. Из результатов явствует, что две трети количества азота, задержанного организмом во время беременности, измеряется в воспроизводительных органах или в плодах. Величина излишка задержки в период 21-дневной беременности составляет у молодых животных 1207 мг, а у старых животных — 1575 мг. Среди исследованных органов процентное содержание азота матки и сосков молочных желез, печени и икроножной мышцы было значительно ниже, чем у небеременных контрольных животных. За исключением величин содержания азота в сосках молочных желез автор получил подобные результаты также в случае старых беременных животных. Как в воспроизводительных так и в вегетативных органах у беременных животных получалось только абсолютное повышение количества азота, которое проявлялось в значительном увеличении веса отдельных органов.

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Acta Biol. Hung. **15** (1), 39—44 (1964)

HISTOCHEMICAL AND BIOCHEMICAL STUDIES ON MUCOPOLYSACCHARIDE CONTENT OF THE ORGANS OF FRESH-WATER MUSSEL (ANODONTA CYGNEA L.)

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(Received November 30, 1963)

Synopsis

The mucopolysaccharide content was investigated in the organs of fresh-water mussel *(Anodonta cygnea* L.) with biochemical and histochemical methods. It was demonstrated by histochemical techniques that the PAS-positive substances in the different tissues of mussel (except the muscle tissues) are neutral mucopolysaccharides, whereas in the glands acid mucopolysaccharides were detectable. A high hexosamine content was found in the tissues, several times greater than in the tissues of mammals. It is assumed that the reverse relationship between oxygen supply and mucopolysaccharide content of tissues as suggested by Krompecher and co-workers exists not only under experimental and pathological conditions, but is detectable also in the relatively poorly oxigenized tissues of fresh-water mussel standing at a lower level of phylogeny.

Introduction

Recent investigations by Krompecher and associates [1, 4, 5, 9, 10, 11, 12, 13, 14, 15) demonstrated that if the blood supply of tissues is damaged by effects, either physiological (pressure on regenerating granulation tissue on the articular surface) or pathological (tuberculous tissue, carcinoma, silicosis, ulcus cruris, myxoedema, burning, freezing) a more intensive mucopolysaccharide formation takes place at a certain degree of oxygen deficiency. This manifests itself by histochemical methods in the appearance of a substance, which is PAS-positive, becoming PAS-negative after acetylation, and may be rendered again PAS-positive subsequently by saponification. This substance gives no metachromasia, produces methylene blue extinction only at high pH values, is indigestible by substances, as pepsin, trypsin, hiase, diastase or saliva. As biochemical studies show, this mucopolysaccharide formation is accompanied by a simultaneous increase in hexosamine content of tissues. According to Krompecher [12, 13] this is presumably due to the adaptation of tissues to a poorer oxygen supply. For investigating this problem more intensively it was suggested to examine the tissues of an animal which is living constantly in hypoxibiotic circumstances, all the more, because Gabe [3],

for instance, succeeded to demonstrate in a whole series of lower animals, and among them also in the molluscs a substance complying with the majority of the above histochemical requirements. On basis of these considerations it was decided to perform experiments on fresh-water mussel, which has according to SALÁNKI [19] an oxygen consumption of 0.6—1.6 mg/l hour at 10—15° C (in case of 14—16 cm long specimens).

Material and methods

These investigations were carried out between April and September on 12—18 cm long specimens of fresh-water mussel *(Anodonta cygnea L.)* originating partly from the Hortobágy river and partly from Lake Balaton. Foot, mantle, gill, adductor muscle, syphon, heart and kidney of the animals were investigated histochemically and biochemically.

Histochemical methods

The above mentioned organs were put into Susa or Gendre fixatives [18] and after washing and dehydration they were embedded into paraffin and serial sections of 5—7 μ were prepared. Besides the routine stainings (Haemalaun-chromotrop 2R, Azan, iron trioxyhaematein-picrofuchsin according to Hansen) the following histochemical reactions were carried out:

(1) The reaction according to RITTER and OLESON [17] which unites Hale's and the PAS reaction. The reagents were prepared according to the original description by RITTER and OLESON. PAS reaction was checked for its correctness by Ritter-Oleson reaction, omitting the treatment with periodic acid.

(2) After acetylation Ritter-Oleson reaction [16].

(3) Acetylation + saponification + Ritter-Oleson reaction.

(4) Lipid extraction according to the method employed by KEILIG [6] performed with chloroform/methanol, ether, acetone through a period of 24 hours at 60° C, with subsequent embedding into paraffin and Ritter-Oleson reaction.

(5) Pepsin-digestion for 2 hours at 37° C in 0.02 N HCl containing 2 mg/ml crystalline pepsin. A similar incubation in 0.02 N HCl served as control.

(6) Trypsin-digestion for 2 hours at 37° C in a phosphate buffer at pH **8.**0 containing 1 mg/ml trypsin.

(7) Hyaluronidase-digestion for 16 hours at 37° C in "Hyason" (Organon) solution of 150 unit/ml concentration prepared in its original solvent.

(8) Metachromatic reaction with toluidine blue of 0.02 per cent concentration [16].

(9) Methylene blue extinction in Walpole buffer at 1.42; 3.09 and 4.76 pH values [16].

(10) Sulphation [7,8] in H_2SO_4 of a specific gravity of 1.80 for one minute, followed either by metachromatic reaction with toluidine blue of 0.02 per cent concentration or by methylene blue extinction at 1.42 pH value.

(11) Gendre fixation [18] followed by paraffin embedding with subsequent Ritter-Oleson reaction.

(12) Salivary-digestion for two hours at 37° C on material fixed in Gendre fixative followed by Ritter-Oleson reaction [16].

Biochemical analysis

Hexosamine contents in the tissues were determined by BOAS's [2] method. Dry tissue-powder was prepared of the removed organs with acetone. An about 0.03 g portion of this tissue powder was hydrolized for 4 hours in 4N HCl. Disturbing chromogenes were removed with Dowex 50 × 8, mesh 200—400 synthetic resin from the hydrolizate adjusted to 0.8 pH by suitable dilution. The hexosamines were eluated from resin in acid milieu and after neutralization were measured by ELSON-MORGAN's method (at the same hydrogen ion concentration

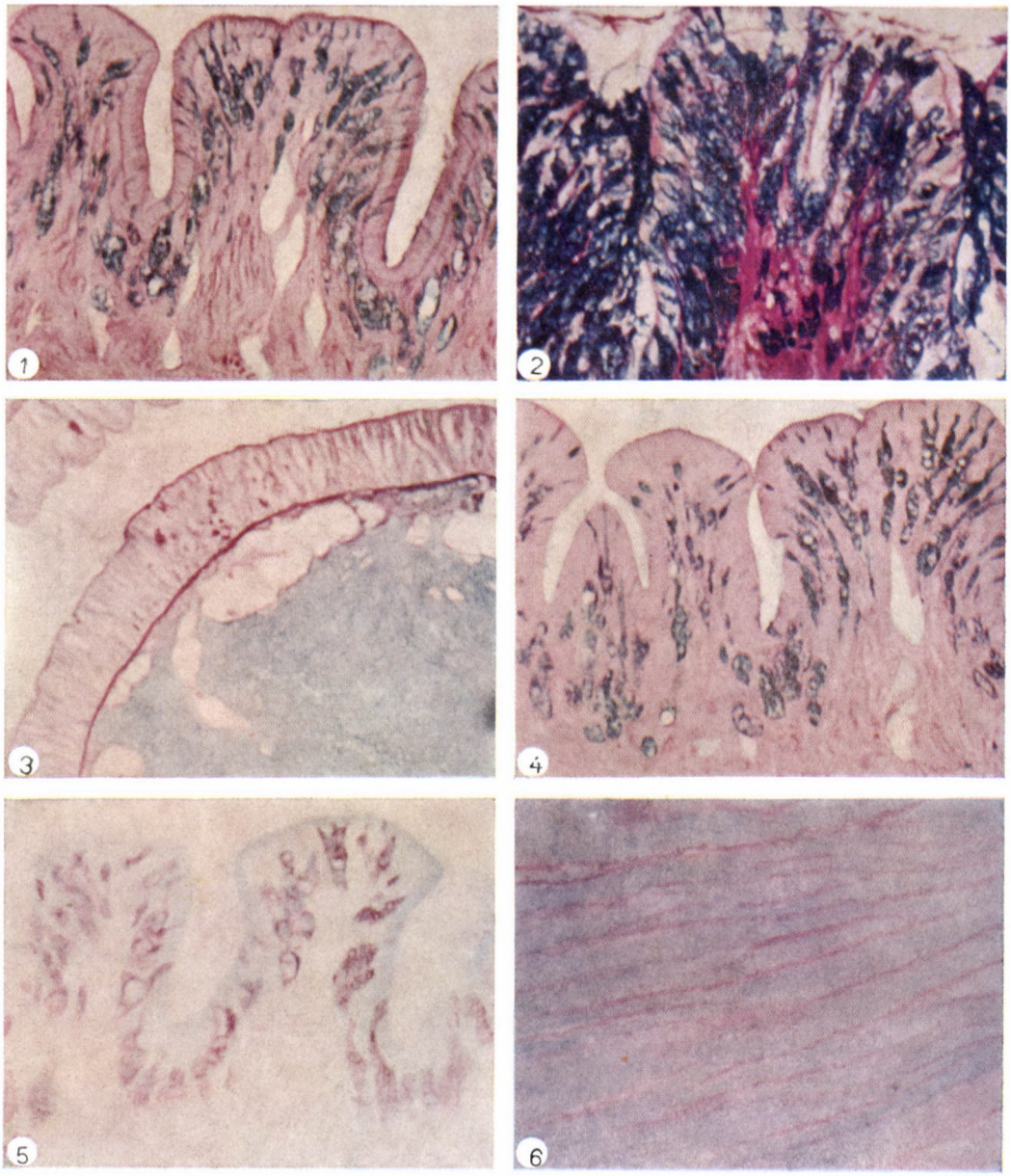

Fig. 1. Superficial part of foot. Susa-fixation, paraffin embedding, Ritter-Oleson reaction. × 200. *Fig. 2.* Superficial part of foot. Lipid-extraction according to Keilig in chloroformmethanol, paraffin embedding. Ritter-Oleson reaction. × 200. *Fig. 3.* Section of alimentary canal. Fixation in Susa, paraffin embedding. Trypsin-digestion + Ritter-Oleson reaction. × 200. *Fig. 4.* Superficial part of foot. Fixation in Susa, paraffin embedding. Pepsin-digestion + Ritter-Oleson reaction. × 200. *Fig. 5.* Superficial part of foot. Fixation in Susa, paraffin embedding. Metachromasia with toluidine blue. Metachromasia only in the glands. × 200. *Fig. 6.* Longitudinal section of adductor muscle. Gendre fixation, paraffin embedding. Salivary-digestion + Ritter-Oleson reaction. PAS-positive substance remained only in the connective tissue between muscle fibres. × 400.

as the standards). Glucosamine HCl was used as standard. Acetylation was performed with acetylacetone for 45 minutes on a water-bath of 89—92° C. Differences in intensity of colour developed with Ehrlich reagent were measured with Pulfrich step-photometer.

Results

Histochemical results

In the tissues of the examined organs of fresh-water mussel a diffuse positive PAS-reaction was observed in general. PAS-positive were the following organs: connective tissue, basal membrane beneath the epithelium, perineurium, wall of vessels, muscles, heart muscle, the superficial part of cylindrical epithelium covering the surfaces and the granulous substance in the epithelium of kidney. As an example a section of the foot is presented in Fig. 1.

Hale-positive were the following regions: mucus gland of foot, submucosa of intestinal canal in the region of typhlosole, the unicellular endoepithelial glands in the epithelia of secretory ducts of sexual organs, of oral lobe, mantle, gill and syphon.

There is no PAS-positive reaction when the periodic acid treatment is omitted. Similarly, the PAS reaction is not positive after acetylation, but it becomes positive again on the effect of saponification subsequent to acetylation.

PAS-positive reaction remains unchanged in the above-mentioned regions after lipide-extraction performed by Keilig's method (Fig. 2).

The results obtained were almost equal when carrying out Ritter-Oleson's reaction either on sections digested with ferments and on their controls or on undigested sections (Fig. 3 and 4).

PAS-positive sections did not produce metachromasia with toluidine blue (Fig. 5) and methylene blue extinction was also observed only at pH 4.76.

After sulphation a more extensive toluidine blue metachromasia and a strong methylen blue extinction at pH 1.42 as well were obtained in PAS-positive regions.

Equal PAS-positive reaction was obtained in sections fixed in Gendre and Susa fixatives, the PAS-positive substance, however, disappeared both from the muscles of foot and from the adductor muscle after salivary-digestion (Fig. 6).

The reactions in Hale-positive regions did not change even after acetylation. Glycogene was not observable in these regions and the reaction remained positive even after lipid-extraction. Hale-positive substance was not digested by hyase, pepsin, trypsin (Figs. 3 and 4) and saliva. Metachromasia with toluidine blue was observed only in Hale-positive regions (Fig. 5), and methylene blue extinction was obtained in the same region also at a 1.42 pH value.

Biochemical results

The averages and standard deviations of results obtained by hexosamine determinations in the organs of *Anodonta cygnea* using the technique of BOAS are as follows (the figures after the name of organs indicate the number of determinations).

foot (6)	674.6±53.5 mg%
kidney (5)	700.8±32.4 mg%
gill (5)	563.5±36.6 mg%
heart (5)	740.3±33.4 mg%
mantle (6)	632.9±89.0 mg%
syphon (5)	658.6±25.0 mg%
adductor muscle (5)	307.4±31.2 mg%

Discussion

The findings obtained on acetylation and subsequent saponification establish the carbohydrate character of PAS-positive substances present in the connective tissue, basale membranes, superficial part of epithelium and in the epidermis cells of the kidney. This substance cannot be regarded as a protein-like substance because it is indigestible with pepsin and trypsin, it cannot be a lipid either, due to the ineffectiveness of lipid extraction. It is assumed, on basis of generally accepted criteria in histology, that the PAS positive reaction observable in the above regions is due to the presence of neutral mucopolysaccharides. This supposition is confirmed by the following findings: ineffectiveness of salivary digestion on PAS-positive reaction, methylene blue extinction only above pH 4, toluidine blue metachromasia only after sulphation.

The PAS-positive substance detected in adductor muscle, heart muscle, and the muscles of the foot can be identified as glycogen by its different histochemical reactions.

The Hale-positive substances are presumably acid mucopolysaccharides. This supposition is supported by the following findings: behaviours on acetylation and subsequent saponification, results of both digestive experiments with different enzymes and of lipid-extraction, the gamma-metachromasia with toluidine blue and the methylene blue extinction at low pH values.

Since the hexosamine investigations were performed with homogenized organs, the evaluation of the data obtained is not easy, because the relative proportion of the single tissues in the organs is unknown. Nevertheless it is regarded as a valuable finding that the biochemically detected hexosamine content is parallel with the histochemically determined presence of mucopolysaccharides, e.g. if only a minimum quantity of hexosamine is present in the

adductor muscle, the amount of histochemically demonstrable mucopolysaccharides is low and on the contrary when the quantity of the former is high, then the quantity of the latter is similarly high. The hexosamine content measured in the organs of fresh-water mussel is much higher than in the case of mammals (rat skin 254.5 mg% [14], human skin 239.7 mg%).

The reverse correlation between oxygen supply and mucopolysaccharide content of tissues suggested by KROMPECHER and co-workers is supported by these findings. Further detailed studies on metabolic processes are needed, however, for the correct understanding of this relationship.

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HISTOCHEMISCHE UND BIOCHEMISCHE UNTERSUCHUNGEN ÜBER DEN MUCOPOLYSACCHARIDGEHALT DER ORGANEN DER SÜSSWASSERMUSCHEL (ANODONTA CYGNEA L.)

Die Autoren haben den Mucopolysaccharidgehalt der Organe der Süsswassermuschel mit histochemischen und bichemischen Methoden geprüft. Es wurde bestätigt, dass die in den Geweben befindliche PAS-positive Substanz mit Ausnahme der Muskelgewebe histochemisch neutralen Mucopolysacchariden entspricht, und dass in den Drüsen saure Mucopolysacchariden nachweisbar sind. Der Hexosamingehalt der Organe ist hoch, vielfach höher als in den Geweben der Säugetiere. Das verkehrte Verhältnis zwischen Mucopolysaccharidgehalt und Oxygenversorgung der Gewebe, welches von KROMPECHÉR und Mitarbeitern aufgestellt wurde, scheint nicht nur unter experimentellen und pathologischen Bedingungen gültig zu sein, sondern auch im Falle der mit Oxygen schlecht versorgten Gewebe der auf einer niederen Stufe der Phylogenesis stehenden Muschel.

ГИСТОХИМИЧЕСКОЕ И БИОХИМИЧЕСКОЕ ИЗУЧЕНИЕ СОДЕРЖАНИЯ МУКОПОЛИСАХАРИДОВ ОРГАНОВ БЕЗЗУБКИ (ANODONTA CYGNEA L.)

Авторы изучали содержание мукополисахаридов в органах беззубки (*Anodonta cygnea* L.) при помощи гистохимических и биохимических методов. Было установлено, что ПАШ-позитивная субстанция, обнаруженная в тканях беззубки, кроме мышечнай ткани, гистохимически соответствует неутральным мукополисахаридам, а в железах можно выявить кислые мукополисахариды. Содержание гексозамина в органах беззубяи во много раз выше, чем в органах млекопитающих.

Авторам кажется, что теория *Кромпехера* и его сотрудников, согласно которкй снабжение кислорода тканей обратно пропорционально содержанию мукополисахаридхв в этих тканях, справедливо не только при экспериментальных и патологических условиях но останется в силе и для относительно плохо снабженным кислородом тканей беззубки которая находится на низшей ступени филогенетического развития.

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Acta Biol. Hung. **15** (1), 45—57 (1964)

STUDIES ON THE INHERITANCE OF A TYPE OF FASCIATION IN MAIZE (ZEA MAYS L.) EARS

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(Received December 15, 1963)

Synopsis

The inheritance of fasciation of the maize ears has been investigated in dialle crosses (P, F_1, F_2, B_1, B_2, F_3) of the following lines selfed for 7—8 generations: a 4-(P4) and an 8-(P8) rowed flint, a 12-(P12) and a 17.3-rowed (on the average; P18) dent, and a 39-rowed (on the average; P40) pop corn with fasciated spheroid ears. Individuals with fasciated ears occurred in all generations of the cross P18 × P40 with a higher frequency than 50%, while in the other combinations of P40 there were no fasciated ears in the F_1 and B_1. However, they occurred with a frequency below 50% in the F_2, B_2 and F_3 generations showing some relation with the kernel row number of the non recurrent parental lines. A genetic model was developed in a fairly good agreement with the data of the experiment. Line P18 probably deviates from line P40 in two factors associated also with suppressors; between lines P18 and P12 there may be a difference of only one dominant factor, while the differences between the lines P12 and P8 as well as between lines P12 and P4 are complicated. The data show definitely that not only the higher kernel row numbers are likely to be brought in relation with the fasciation, because even the P8 and P4 lines with low kernel numbers contain inhibiting dominant alleles. The fasciation and the branching of the maize ear are inherited independently of each other.

Introduction

The inheritance of teratological phenomena has been investigated by DEVRIES [5] as early as the beginning of the century. He concluded that they are due partly to heredity and partly to environmental factors. Among others he has also studied fasciation. The relevant literature has been recently summarized by WHITE [20]. The fundamental cause of the appearance of fasciation, observed so far in more than one hundred different plant families, seems to be a disturbance in the metabolism of the plant; as a consequence of an abundance of nutrient supply accumulation of energy results which leads to the abnormal tissue formation. Anatomical investigations indicate that the evolution of various types of fasciation in contrast to earlier assumption, must be attributed to abnormal development of the growth region rather than the united growth of several such regions.

Primarily on genetical grounds, WHITE classifies the different fasciations as follows:

1. Inherited fasciation.

2. Fasciation due to various natural environmental causes, which are not inherited.

3. Spontaneous fasciation of unknown origin propagated asexually, but, so far as known, not through seed.

4. Fasciation induced artificially by known procedures which possibly in some cases have been asexually propagated.

5. Fasciation which has not been investigated experimentally as yet and which remains unclassified as to transmissibility.

There are many incidences of inherited fasciations and all cases reported arose as mutations. The most common is the monogenic recessive inheritance, but dihybrid and trihybrid segregation as well as dominant and intermediate inheritance also occur.

Fasciation in maize ears has been investigated by many workers. The normal maize ear was considered as a type of fasciation by COLLINS, CUTLER and WEATHERWAX; DEVRIES and HUS and MURDOCK range it to the eversporting category of mutants, while EMERSON and HAYES [19], EMERSON and WHITE find true breeding races in which the intensity of expression of the character frequently fluctuates. Fasciation may behave either as recessive, or as partially dominant, or as dominant character. In most cases the segregation is monogenic, but the possibility of dihybrid segregation is also given. In WEIJER's catalogue of genetic maize types it is recorded as "fs. — Classification apparently fair. Viability good. Chrom. unknown" [18].

Recently the influence of growth conditions on the formation of organs was investigated by JANUŠEVIČ [8] who states that optimum environmental conditions may result in the fasciation of the ears.

Formerly, fasciation was exclusively qualified as a teratological phenomenon and generally attributed to the fusion of homologous organs. SZÁSZ [16] for example mentions it in the chapter "Cohaesio" of the part "Fusion of the organs" of his book on the teratology of plants published in 1878. FILOV [6] recently states that fasciation is in reality no teratological phenomenon, but the natural process of progressive evolution influenced by cultivation, and several plants species, among others also maize, are known only in fasciated forms. But he attributes fasciation still to fusion of homologous organs [7]. Earlier data as well as the recent ones of SHAVAROV [13] on cross-sections of Leucanthemum vulgare stems, and illustrations of *Pisum sativum* growing points by LEBEDEVA [9] definitely support the theory, that no fusion is involved in fasciation. WEATHERWAX [17] concludes: "It is unfortunate that the term fasciation has been applied to these (cases, i.e. flattening of the tip of the ear in various ways); it carries with it the literal meaning of a bundle of sticks . . . A true fasciation, as defined botanically is simply an enlarged, flattened, and often branched structure, which has the appearence of several fused branches."

It is known that the factor inducing the abnormal growth of the growing point resulting in fasciation in peas is a tryptophan derivate. Although the

fasciation is genetically determined already in the embryo, it is only possible to demonstrate a quantitative difference in the auxin content, and even that only after a certain period of development [15].

Material and methods

The study of the inheritance of kernel row number was the aim of our experiments [3], however, the investigations were extended to other characters, and among them to fasciation. Diallel crosses were performed between five lines selfed through 7—8 generations: a 4-(P4) and an 8-(P8) rowed flint corn, a 12-(P12) and a 17.3-rowed (on an average; P18) dent corn, and also an average 39-rowed (P40) pop corn with fasciated, spheroid ears. The suitable generations (P, F_1, F_2, B_1, B_2, F_3) were produced and comparative experiments established of two years at the same locality in a 4 × 4 "Latin rectangle" [12] arrangement. The number of the investigated individuals and those with fasciated ears are reported in Table 1 and Table 2 according to combinations and generations. The third progeny (F_3) was produced in the first year of the comparative experiments and so it was included only in the second year experiments.

The ears were grouped on the basis of their abnormalities first in several groups, then in three, and finally in two classes. Considering the grouping of SĂVULESCU and ZAHARIADI [14], fasciation, branching at the top and furcation of the ears were all considered as fasciations, in accordance with the authors who also regard them to be related phenomena; the ramifications which arose from the basal part of the ears or developed from axillary buds among the husks were considered as branching. To control whether the classification was correct or not, the interdependence of the inheritance of fasciation and branching was tested; the Chi^2 test proved significantly the independent inheritance (Table 3) as it was suggested already in a previous report [4].

The data of the two years are rather fluctuating, but the variance analysis of the values expressed in % and transformed does not show any significant deviation ($P = 0.2—0.3$), whereas variations attributed to combinations and generations are fairly reliable ($P = 0.001$). For that reason the fluctuation observed between the two years was considered as error and the further analysis was made with the combined data for the two years.

A genetic model was established and the experimental data were compared with the values estimated on the basis of the model (Table 4). The F_3 averages were ascertained on the basis of the progenies of relatively few F_2 plants (Table 2) and thus instead of the Chi^2 test applied to the other generations, the following method was employed: On the basis of the assumed model the expected classes of the F_3 generations were established with the corresponding frequencies; then the class was chosen at which the whole F_3 generation could be divided in two in such a way, that the average of the part reaching to the respective class should be lower, and the average of the part containing the class itself higher than the average obtained by experiments; the experimental data corresponding to the two limit values of the respective class raised to the power according to the number of the F_3 families counted, gave the limit values of probability. (The averages of the experimental data were in each case lower than the theoretical ones; in the opposite case the order had to be inverted.) The statistical analysis was made after MATHER [11].

Results

In all the F_2, B_2 (F_1 × P40), and F_3 generations of the crosses with the P40 line as recurrent parent, individuals with fasciated ears occurred in the entire diallel cross in quantities to be ascribed simply to environmental effects, except for the F_1 and B_1 generations of the P18 × P40 cross, where they occurred in high percentages. At first a mistake was suspected but repeated crosses produced equally an F_1 generation with fasciated ears. (Fig. 1 shows characteristic ears of the parents and the F_1 generation; Fig. 2 such of later generations.) It appears clearly from the data obtained, that there is no simple

Table 1

Distribution of the individuals with fasciated ears in the P, F_1, F_2, B_1 ($[P_1 \times P_2] \times P_1$ *and* B_2 $[P_1 \times P_2] \times P_2$) *generations of a 5 × 5 diallel cross.*
(n = number of individuals counted; fs = number of individuals with fasciated ears)

Parental lines		P4		P8		P12		P18		P40	
	Item	n	fs	n	fs	n	fs	n	fs	n	fs
P4	F_1	148	1	150	—	144	—	149	—	156	—
	F_2			296	—	307	—	303	—	311	22
	P										
	B_1			194	—	194	—	192	—	205	—
	B_2			208	—	211	—	205	5	204	72
P8	F_1					151	—	156	—	154	—
	F_2					310	—	330	2	305	35
	P			153	—						
	B_1					199	—	213	—	212	—
	B_2					196	—	210	—	224	41
P12	F_1							153	—	150	1
	F_2							308	—	322	55
	P					111	—				
	B_1							206	—	205	1
	B_2							203	—	209	85
P18	F_1									147	141
	F_2									314	209
	P							158	2		
	B_1									207	112
	B_2									211	156
P40	P									134	134

monohybrid, or dihybrid segregation and even the genotypes of the lines employed are different not only in the kernel row number, but in the segregation ratios of fasciation, too.

Starting with the line showing the highest number of individuals with fasciated ears in the progenies, the approximative genotype for each line was established:

P40	aa	Su—A	Su—A	bb	su—B	su—B	cc	dd	su—D	su—D	ee	ff
P18	AA	su—A	su—A	BB	Su—B	Su—B	cc	dd			ee	ff
P12	AA	su—A	su—A	BB	Su—B	Su—B	CC	dd			ee	ff
P8	AA	su—A	su—A	BB	Su—B	Su—B	CC	dd			EE	FF
P4	AA	su—A	su—A	BB	Su—B	Su—B	CC	DD	Su—D	Su—D	ee	ff

Table 2

Frequency of individuals with fasciated ears in the F_3 generations of a 5 × 5 diallel cross

Crosses	Number of families	Individuals counted	Individuals with fasciated ears	
			number	%
P4 × P8	7	207	0	0
	1	34	1	2.94
	8	241	1	0.41
P4 × P12	9	269	0	0
	1	35	1	2.86
	10	304	1	0.33
P4 × P18	10	319	0	0
P4 × P40	6	200	0	0
	1	34	1	2.94
	1	39	5	12.82
	1	28	5	17.85
	1	31	15	48.38
	10	332	26	7.83
P8 × P12	10	333	0	0
P8 × P18	9	313	0	0
	1	40	3	7.50
	10	353	3	0.85
P8 × P40	6	211	0	0
	1	31	2	6.45
	1	38	3	7.89
	1	30	3	10.00
	1	26	4	15.38
	10	336	12	3.57
P12 × P18	10	337	0	0
P12 × P40	2	74	0	0
	1	32	1	3.13
	1	31	1	3.23
	1	36	2	5.56
	1	28	2	7.14
	1	34	3	8.82
	1	32	4	12.50
	8	267	13	4.87
P18 × P40	1	34	0	0
	1	34	7	20.58
	1	38	15	39.47
	1	35	15	42.85
	1	34	15	44.11
	1	34	19	55.89
	1	35	21	60.00
	1	34	21	61.76
	1	34	22	64.70
	1	37	24	64.86
	1	36	24	66.67
	1	29	20	68.96
	1	36	25	69.44
	1	27	19	70.37
	1	37	27	72.97
	1	39	32	82.05
	1	32	27	84.37
	17	585	333	56.92

Table 3

Relationship between fasciation and branching of the corn ears

Cross	Number of individuals								Probability of independent inheritance %		
	total		fasciated		branched		branched and fasciated				
	1958	1959	1958	1959	1958	1959	1958	1959	1958	1959	1958+59
F_1											
P18×P40	77	70	72	69	52	57	49	56	95—90	98—95	90—80
F_2											
P4 ×P40	206	105	19	3	22	9	2	—	100—99	70—50	90—80
P8 ×P40	213	92	25	10	6	1	1	—	80—70	80—70	90—80
P12×P40	213	109	45	10	57	11	10	2	70—50	50—30	95—90
P18×P40	207	107	128	81	109	51	63	38	70—50	90—80	70—50
B_1											
(P18×P40)P18	118	89	60	52	50	58	27	39	80—70	50—30	50—30
B_2											
(P4 ×P40)P40	117	87	26	46	19	6	6	2	50—30	30—20	80—70
(P8 ×P40)P40	137	87	23	18	11	1	2	—	95—90	70—50	90—80
(P12×P40)P40	120	89	48	37	22	18	10	9	70—50	70—50	70—50
(P18×P40)P40	119	92	77	79	58	23	35	22	70—50	70—50	70—50
Total	1527	927	523	405	406	235	205	168	100—99	95—90	100—99

In the system the genotypes aa and A Su-A — bb and B Su-B — cc — dd and D su-D su-D — ee ff and EF have fasciated ears.

In spite of the good agreement of the values estimated by the model with those obtained from experiments (Table 4), these hypothetical genotypes

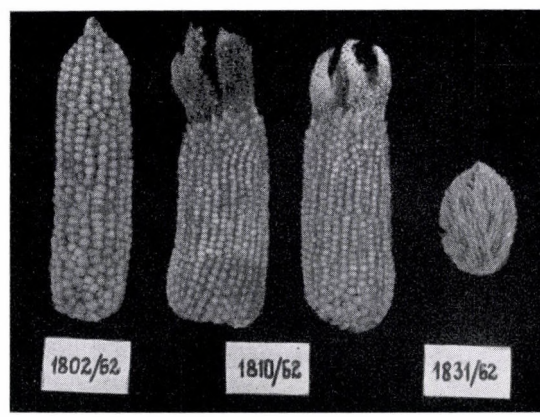

Fig. 1. Ears of the two parental lines P18 (left) and P40 (right), and their F_1 generations (the two ears in the middle)

Table 4

Comparison of experimental and estimated data of the crosses of a line with fasciated ears and four lines with different kernel row numbers

Population	Generation	Number of individuals with fasciated ears		Probability of coincidence %
		experimental	estimated	
P4	P	0.68	0	95—90
P8	P	0	0	100
P12	P	0	0	100
P18	P	1,27	0	95—90
P40	P	100.00	100.00	100
	F_1	0	0	100
	F_2	7.07	7.22	100—99
P4×P40	B_1	0	0	100
	B_2	35.29	28.13	5—1
	F_3	7.83	13.42	42—47
	F_1	0	0	100
	F_2	11.47	10.31	50
P8×P40	B_1	0	0	100
	B_2	18.30	18.75	90—80
	F_3	3.57	11.62	6—9
	F_1	0.67	0	95—90
	F_2	17.08	16.50	80—70
P12×P40	B_1	0.49	0	95—90
	B_2	40.66	37.50	50—30
	F_3	4.87	22.02	1.3—6.6
	F_1	95.91	100.00	70—50
	F_2	66.56	66.01	90—80
P18×P40	B_1	54.10	50.00	30—20
	B_2	73.93	75.00	80—70
	F_3	56.92	58.72	2.7—100
				47—51*

* estimated on individual basis

with suppressors and complementary factors are rather artificial and in order to stress this, the usual symbols "fs" or "fa" for fasciation were omitted.

According to the model established for the P18 × P40 cross containing two factors with suppressors and in which the fasciation is recessive, the expected distribution of the individuals with fasciated ears for the generations

are: $F_1 = 100\%$, $B_1 = 50\%$, $B_2 = 75\%$, and $F_3 = 58.72\%$. The data coincide with the results obtained experimentally and the probability of coincidence for the generation showing the greatest difference is between 0.3 and 0.2. By adding another dominant factor, F_1 and B_1 will not be fasciated, and the percentage of individuals with fasciated ears will diminish in the F_2 to a quarter and in the B_2 to the half. After this addition, the data obtained do well agree

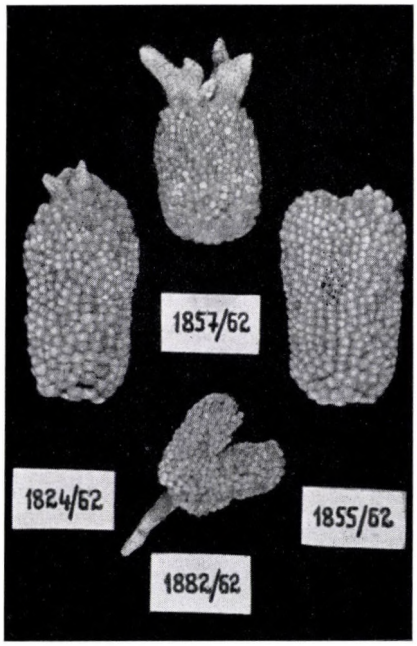

Fig. 2. Some characteristic ears from late generations of the P18 × P40 cross

with the P12 × P40 cross, except for the F_3 generation consisting only of 8 families. If a further dominant factor with recessive suppressor is supposed, the value of F_2 decreases to its 7/16, while the value of B_2 falls only to its $\frac{3}{4}$. F_2 and F_3 coincide very well with the corresponding generations of the cross P4 × P40, while B_2 does not, but falls between the values of the two years (Table 3). By supposing a complementary system instead of the dominant factor with a recessive suppressor, in which the two dominant factors, if both are present, neutralize their mutually inhibitory effects, the diminution in F_2 will be of 10/16, while in B_2 it will be of $\frac{1}{2}$. This is in good agreement with the cross P8 × P40.

Taking in consideration all the data obtained, the values of the F_3 generations in the cross P18 × P40 are slightly while in the others rather low. F_2 and B_2 generations of line P4 show in the two years a divergence of 100%, the direction of this divergence is opposite in these two generations.

Discussion and conclusions

According to the combined data of our comparison, which included 928 individuals with fasciated, 641 individuals with branched, and among them 373 with both fasciated and branched ears, the two characters are inherited independently of each other with a probability of 99—100%. It could be established that the fasciation as well as the ear furcation, shown in Fig. 32 of WEATHERWAX [17] as a non inherited anomaly of the maize ears, and also shown in our Fig. 2, may be classified as fasciated ears. Its occurrence is so rare, that this could not cause any alterations of the results and thus the study of its inheritance was impossible.

Our experiments explicitly show that the fasciation of the P40 line used belongs to the "inherited fasciation" group, according to the classification of WHITE [20], though its inheritance is not of simple monohybrid or dihybrid type, but more complicated, and differs from cross to cross according to the parents with different kernel row numbers. The study of the experimentally best investigated type of fasciation in "*Pisum umbellatum*" ("Mummy pea" or "Crown pea") revealed quantitative differences in the content of four auxins between the fasciated and the nonfasciated types, and fasciation could be even induced by two auxins [15]. Peculiarly, the mode of inheritance was, notwithstanding, according to most data, monohybrid recessive, only incomplete penetrance and variable expressivity as well as modifiers caused some deviations in the segregation ratios [10]. Assuming that fasciation is produced by auxin effects also in our case, the following physiological processes are to be taken into consideration, each of which may stand under the control of different alleles:

1. Production of auxin precursor
2. Transformation of the precursor into auxin
3. Oxidation of auxin
4. Translocation of auxin.

It is well known, that the normal development depends on a certain optimum auxin level as well as on the qualitative properties of the growth factors. Although the fasciated ears may show a simple monohybrid or dihybrid segregation, a complicated inheritance is more probable.

In accordance with earlier data and with our recent investigations, the fasciation of maize ear has more than a single type and the P40 parental line probably represents an extreme type only. This opinion is supported by our observations, that the first generation of P18 × P40 shows almost completely fasciated ears, however, none out of 10 individuals of the first generation of the cross between P18 and a line of an other type of fasciation has fasciated ears. Accordingly of course, our data are valid in the strict sense only for the fas-

ciation of the P40 type that is probably the same as the type shown on Fig. 31 of WEATHERWAX [17].

There was a definite relation between the percentages of individuals with fasciated ears and the kernel row number of the nonfasciated parent in the hybrid generations of our material (Fig. 3). While among the investigated five progenies of the cross between P18 with an average of 17.3 kernel row number and P40 even none of the backcross generations showed less than 50% of individuals with fasciated ears, the F_1 and B_1 generations of the lines with lower kernel row number had no fasciated ears and the average values of B

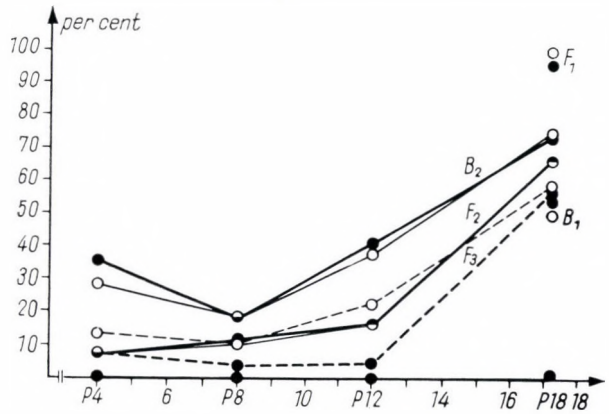

Fig. 3. Relationship between the average kernel row number of the parents and the frequency of the individuals with fasciated ears (ordinate) in a cross of 4 (P4), 8 (P8), 12 (P12) and 17,3 (P18) rowed lines (abscissa) with the fasciated line P40 from Fig. 1 (● = experimental data; ○ = estimated data)

($F_1 \times$ P40) were all below 50%. Therefore we may consider the P40 line as the first step, and the P18 line (showing a complicated inheritance in crosses) as the second step, while the P12 line with a kernel row number of 12 as the third step; this latter differs probably from the P18 line only by a dominant factor inhibiting the fasciation. The fourth step is represented by both P8 with a kernel row number of 8, and P4 with a kernel row number of 4. In the combinations the number of individuals with fasciated ears is further decreasing, but the results cannot be derived from any of the former steps by assuming a simple monohybrid or dihybrid inheritance. Between lines P8 and P4 there is a definite difference, however, there are some difficulties to establish the proper order of their ranking. The percentage of the individuals with fasciated ears were in both years of the experiment higher in the F_2 generation of the P8 cross, while in the B_2 generation it was higher in the P4 combination. Furthermore, the average difference between the corresponding data of the two years of the whole experiment was the smallest in the P8 combinations,

and the greatest in the P4 combinations. P4 line was selfed from the "Florentini" variety with 8 kernel rows and is near to the type with 8 kernel rows, but it is not related to line P8. In spite of its very low variance for kernel row number, the ear of P4 with its abnormal form is to be regarded as a teratological phenomenon [3] and its identification is very difficult.

The averages of the F_3 generations were always lower in each cross, than the values expected from the model based on the other generations (Fig. 3). Unfortunately, the data of the one year experiment and of the F_3 progenies obtained from a small number of F_2 plants except for the P18 $\times$ P40 cross, are not suited for any generalized conclusions. We can assume, that the low averages of the F_3 resulted from the decreased vigor.

The question often arises whether the maize ear is a fasciated form or not. Investigating the homology of the tassel and the ear, ANDERSON [1] states: "The new evidence also suggests quite as clearly that fasciation is only one of several forces and that in North American maize it operates only in ears of more than 8 or 10 rows." FILOV [6] simply mentions that maize is only known in the fasciated form. Our experiments definitely show that individuals with fasciated ears were produced even in the progenies of the crosses of the lines P8 or P4 and the used fasciated line, in spite of their total absence in F_1; consequently even the two lines representing the lowest kernel row numbers known in homozygous state, contain dominant factors which inhibit the manifestation of the character. On the basis of our experiments we cannot answer the question, whether the maize ear is a fasciated form and the forms known generally as fasciated ears are extreme types, or only these extreme types are fasciated. The evaluation of our experimental data was performed on the lines of the generally accepted opinion.

Acknowledgements

The writer is grateful to B. GYŐRFFY for his suggestions and the critical reading of the manuscript; to Mrs. E. CSONKA and M. SÁNTA, and to Mr. I. CZIBULYA for the technical assistance.

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UNTERSUCHUNGEN ÜBER DIE VERERBUNG EINES VERBÄNDERUNGSTYPES BEI MAISKOLBEN (ZEA MAYS L.)

Es wurde die Vererbung der Verbänderung des Maiskolbens in dialleler Kreuzung (P, F_1, F_2, F_3, B_1 B_2) von einer 4- (P4) und einer 8-reihigen (P8) Hartmais-, einer 12- und einer durchschnittlich 17.3-reihigen (P18) Zahnmais-, sowie einer durchschnittlich 39-reihigen (P40) Puffmaislinie mit verbänderten, kugelförmigen Kolben, untersucht. Die Frequenz der Individuen mit verbänderten Kolben war in allen Generationen der P18 × P40 Kreuzung höher als 50%, obgleich in den anderen Kreuzungen von P40 die F_1 und B_1 überhaupt keine Verbänderung aufweisen, und die Häufigkeit in der F_2, B_2, und F_3 unter 50% war, einen gewissen Zusammenhang zeigend mit der Kornreihenzahl der Elternlinien. Für die Demonstration des Zusammenhanges wurde ein, mit den experimentellen Daten in ziemlich guten Einklang stehendes genetisches Modell aufgestellt. Es wird angenommen, dass sich P18 von P40 durch zwei Allelpaare unterscheidet, denen sich je ein Suppressor anschliesst; zwischen P18 und P12 ist eventuell nur eine Differenz von einem dominanten Faktorpaar; bisweilen ist der Unterschied zwischen P12 und P8 sowie zwischen P12 und P4 kompliziert. Die Resultate zeigen eindeutlich, dass nicht nur die höhere Kornreihenzahlen aufweisende Linien mit der Verbänderung in Zusammenhang stehen, sondern auch die Linien P8 und P4 mit niedriger Kornreihenzahlen über die Verbänderung verhindernden dominanten Allele verfügen.

Die Verbänderung und die Verzweigung des Maiskolbens vererben sich voneinander unabhängig.

ИЗУЧЕНИЕ НАСЛЕДОВАНИЯ ОДНОГО ТИПА ФАСЦИАЦИИ ПОЧАТКА КУКУРУЗЫ (ZEA MAYS L.)

В диаллильном скрещивании (P, F_1, F_2, F_3, B_1, B_2) линий кукурузы 4-х (P4) и 8-и (P8) рядковой гладкой, 12-ти (P12) и в среднем 17,3-и (P 18) рядковой зубовидной, а также линии лопающейся кукурузы (P40), содержащей в среднем 39 рядков в початке и обладающей фасциированным шарообразным початком, изучалось наследование фасциации початков. Особи с фасциированными початками во всех потомствах скрещивания P18 × P40 возникали с частотой выше 50%, в то время как в потомствах F_1 и B_1 остальных ком-

бинаций линии P40 вообще не встречалось, в потомствах F_2, B_2 и F_3 встречались с частотой ниже 50%, показывая при этом определенную связь с числом рядков в початке родителей. Для демонстрирования указанных связей построена генетическая модель, довольно хорошо совпадающая с экспериментальными данными. Линия P18 отличается от линии P40 вероятно двумя парами аллельных генов, к которым относятся также и супрессоры, а отличия между линиями P12 и P8, а также P12 и P4 являются тоже сложными, в то время как между линиями P18 и P12 по-видимому имеется разница только в одном доминирующем тормозящем факторе. Полученные данные определенно показывают, что с фасциацией могут быть связаны не только линии с более высоким числом рядков в початке, так как линии P8 и P4 с низким числом рядков тоже содержат доминирующие аллели, тормозящие возникновение фасциации. Фасциация и ветвление початков кукурузы передаются по наследству независимо друг от друга.

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Acta Biol. Hung., **15** (1), 59—64 (1964)

THE ANALYSIS OF A STREPTOMYCES CHARTREUSIS POPULATION

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(Received January 3, 1964)

Synopsis

The cultural and physiological-morphological analysis of a *Streptomyces chartreusis* rendsina-population, identified serologically with IPV 554x (BALDACCI) authentic culture, has definitely shown the unstability of several characteristic properties previously regarded as determinant from a differential-diagnostic point of view. Within the same population of a single ecotype, the presence of chromogenic and achromogenic (melanin-positive and -negative), pigmented and colourless variants, possessing different antibiotic spectra and activity, inactive, mutually antagonistic, etc. could be demonstrated.

Introduction

The correct taxonomical determination of microbe-isolations resides in the direct biochemical and cultural-morphological comparison with authentic, first of all with "type" cultures. By these identifications the thorough knowledge of the diagnostic value of the individual features is of utmost importance. This latter can be achieved not only by numerous parallel examinations of inoculations from the same strains [4, 8], or by comparing the properties of strains of different origin though belonging to the same species [1], but rather by the character-analysis [7] of populations properly checked for common origin, a way unfortunately neglected so far, especially with actinomycetes.

The present paper deals with investigations of a *Str. chartreusis* population isolated from a mull-like forest-rendsina soil, with special reference to the stability of some properties suggested by different authors and lately — for the description of actinomycetes for patents — by the "International Committee on Bacteriological Nomenclature Subcommittee on Taxonomy of the Actinomycetes" [2].

Methods

The diluted suspensions of the soil samples (obtained from a region corresponding approximately to a "pedon") were spread on casein-glucose- and asparagine-glucose-agar, in addition to some other special culture media. The purification of strains was carried out by repeated spreading and isolation. The media used for determining cultural properties were as follows: glucose-asparagine agar, glucose-pepton-agar, potato-block and -agar, synthetic agar, oat-flake agar, glycerol-glycine agar, starch agar, nutrient gelatine, pepton agar. The sporulation and mycelium development in microfilm wet-chamber cultures, the morphology of spore

surface in an electron-optical way, were studied. The utilization of C- and N-sources was determined in the synthetic culture medium of Pridham and Gottlieb, while the melanoid pigment formation was detected on tyrosine-yeast extract agar. In order to demonstrate antibiotic activity the strains were pre-cultured on Conn (Na-aspartate) agar in Petri dishes for 5 days, then the test organism was layered in agar medium over the colonies examined. For antibody production rabbits were immunized with crushed mycelium-suspension. The titer of sera and the serological relationship were determined by the indirect haemagglutination method with the modifications recommended for actinomycetes by MÉSZÁROS [6]. The antigens were disengaged by heat-treatment the dilutions of sera were made by the use of "Takatsy spiral-loops" and calibrated dripping-attachments on grooved plexiglass plates. The results of indirect haemagglutination were controlled also by the determination of the inhibition of haemagglutination carried out in a system of 3 haem.-units.

Results

Several thousand isolations were separated from the A_H-horizon of the rendsina in question. From these isolations, 314 strains were identified, on the basis of cultural-morphological comparison, with the species *Str. chartreusis* (Chartreusis series) or qualified as an ecotype of this species. One of the strains was referred to as the "type" of population (strain-R-1-36).

The description of a "rendsina-ecotype" type strain of species *Str. chartreusis* CALHOUN et JOHNSON 1956. Colour of spores: venetus (azureus); substrate mycelium: colourless (flavus-brunus group); sporophore: spira; habitus of the aerial mycelium: lanatus; vegetative mycelium ϕ: 0.6—1.0μ; spore surface: spiny; positive melanine reaction; positive Tresner-Danga reaction; acid formation on bromcresol purple-agar in the presence of lactose, mannose and mannite; growth at 36° C +++, at 42° C —; it peptonizes and slightly coagulates milk; intensive gelatine liquefaction; it does not reduce nitrates; it splits rapidly fats; poor growth on cellulose; it utilizes paraffin poorly, while waxes strongly; it haemolyses; positive tyrosine decomposition; vigorous dextrin-utilization; positive starch hydrolysis; it is inactive towards *E. coli*, *B. subtilis*, *Serratia marcescens*, *Rhizobium meliloti* and *Staphylococcus albus* ; weak antagonistic effect against *Sarcina lutea*, *Saccharomyces carlsbergiensis* and *Aspergillus niger* ; it inhibits strongly *Trichothecium roseum* ; it does not utilise l-sorbose, shows slight if any growth in the presence of inulin, d-sorbite, dulcite and Na-oxalate; positive utilization of d-fructose, l-arabinose, rhamnose, d-galactose, sucrose, maltose, lactose, cellobiose, raffinose, glycerol, d-mannite, mesoinosite, salicine, dextrin, glycogen, and Na-citrate as C-sources; positive utilization of NH_4Cl, $(NH_4)_2CO_3$, NH_4NO_3, $(NH_4)_2SO_4$, urea, glycine, dl-alanine, dl-valine, dl-nor-valine, dl-serine, dl-threonine, cysteine, l-cystine, l-arginine, dl-lysine, dl-aspartic acid, l-asparagine, l-glutamic acid, l-tyrosine, dl-tryptophan, l-histidine, nucleic acid, pepton, and trypton as N-sources; it shows poor growth in the presence of dl-methionine.

As a result of comparing with the authentic culture-material of our collection, the identity of strain R-1-36 with strain IPV 554x of *Str. chartreusis*,

Table 1

The comparison of Str. chartreusis *strains* R-1-36 *and* IPV 554x

	R-1-36	IPV 554x
Colour of spores	venetus	venetus
Habitus of aerial mycelium	lanatus	lanatus
Sporophores	regular multi-wound spirals, spore chains of sympodial branching, long axon	like strain R-1-36
Spore surface	spiny	spiny
Sclerotia, globular sporangia	—	—
d-glucose (control pos.)	++++	++++
Without C-source (control neg.)	—	—
Without N-source (control neg.)	—	—
Fructose	++++	++++
Rhamnose	++++	++++
Raffinose	++++	++++
Sorbite	—	—
Dulcite	—	—
Mesoinosite	++++	++++
$(NH_4)_2CO_3$	++++	++++
NH_4NO_3	++++	++++
$(NH_4)_2SO_4$	++++	++++
Melanine test	++++	++++
Tresner-Danga reaction	++++	++++
Activity against *B. subtilis*	—	—
Activity against *E. coli*	—	—

obtained from Prof. BALDACCI (Milano), could be established. The schematic comparison of the two strains is seen in Table 1.

The titer (++) of immune serum produced by strain R-1-36, with the homologous antigen: 1536. The same value was obtained with antigen of strain IPV 554x. The inhibition of haemagglutination showed the same value with antigens of both strains (24x). The R-1-36 immune serum strongly agglutinated also red blood cells sensitized with the antigen of the closely related strain *Str. viridochromogenes* NRRL-B-1227 (titer: 96x ++++, 384x ++, 768x +). Some further titers with regard to the known authentic strains of other species: *Str. phaeochromogenes* ATCC 3338: —, *Str. venezuelae* ISP 10712: —, *Str. antibioticus* ATCC 8663: —, *Str. olivaceus* INA N-6: 96x ++++, 192x +, *Str. griseolus* ATCC 3325: —, *Str. flaveolus* ATCC 3319: —, *Str. albus* ATCC 3005: —, etc.

As a total, 80 out of the 314 isolated *Str. chartreusis* strains have been submitted to further detailed comparative study. Among these strains 71 produced colourless, while 9 dark brown substrate mycelium. 4 strains formed green, 3 strains reddish-yellow soluble pigment, while 73 strains none. Only one strain proved to be melanine- and Tresner-Danga-negative. Beside 66 inactive strains, 12 strains were effective only against Gram-positive, and 2 strains against both Gram-positive and -negative test organisms. No strain the scope of which was restricted to Gram-negative test-organisms, could be found. The "type strains", more precisely some of their characteristics, of the most important variants are shown in Table 2. The data of Table 2 as well as the examination results of the 80 strains in general, verify that the morphological criteria and the C-source utilization spectrum varied the least within a population. On the other hand, not only the character of the antibiotic spectrum changed, but also mutual antagonism between strains could be detected. No alteration in the serological behaviour of population-variants could be observed (see Table 2).

Discussion

A true picture of the ecological plasticity [5], biochemical character, and even of the taxonomical position of a soil-living variety of a species can be obtained not by studying one single strain but only by means of its population analysis. Melanine formation appears to be one of the four most reliable taxonomical features characteristic of species *Str. chartreusis* and *Str. viridochromogenes* [3]. Furthermore, the demonstration itself of the melanine reaction belongs to characteristics which can most accurately be determined [4]. As it has been shown, strain 2551/R-1-36 is a typical achromogenic form. To range this strain into a new "infrasubspecific subdivision", however, would not be reasonable, since it differs only by one "less" property from a typical *Str. chartreusis* population.

Another characteristic, lately qualified as a first-class taxonomical mark, is the antibiotic spectrum and especially the mutual resistance of strains belonging to the same species or at least subspecies. In fact, ARTAMONOVA and KRASSILNIKOV [1] have separated the *Actinomyces violaceus vicinus* subspecies from *Act. violaceus* on the basis of the antagonism of type strains. The population studied by us contained inactive variants, variants effective only against Gram-positive microbes, and variants effective against both Gram-positive and -negative organisms. Among these variants the occurrence of forms effective against the members of the population has also been observed, etc.

In our opinion, as in the diagnostics of streptomycetes the setting up of the requirements for the direct comparison with authentic strains proved to be a decisive step, likewise it would provide further progress if the real varia-

Table 2

Comparative study of the characteristic variants of a Str. chartreusis population

1, R-1-36 (type)	Sm^-, L, Vn, Sp, Spi, V, Dp^-, H1536, Hh24, Ec^-, Sub^-, Ap^-, M^+, T-D^+, Me^+, Raf^+, So^-, Du^-, Rh^+, Fr^+, Ac^+, An^+, Car^+
2, R-1-36/2037	Sm^b, L, Vn, Sp, Spi, V, Dp^-, H1536, Hh24, Ec^+, Sub^+, Ap^-, M^+, T-D^+, Me^+, Raf^+, So^-, Du^-, Rh^+, Fr^+, Ac^+, An^+, Car^+
3, R-1-36/2040	Sm^b, L, Vn, Sp, Spi, V, Dp^-, H1536, Hh24, Ec^-, Sub^+, Ap^+, M^+, T-D^+, Me^+, Raf^+, So^-, Du^-, Rh^+, Fr^+, Ac^+, An^+, Car^+
4, R-1-36/2043	Sm^-, L, Vn, Sp, Spi, V, Dp^-, H1536, Hh24, Ec^-, Sub^+, Ap^+, M^+, T-D^+, Me^+, Raf^+, So^-, Du^-, Rh^+, Fr^+, Ac^+, An^+, Car^+
5, R-1-36/2068	Sm^-, L, Vn, Sp, Spi, V, Dp^-, H1536, Hh24, Ec^-, Sub^+, Ap^-, M^+, T-D^+, Me^+, Raf^+, So^-, Du^-, Rh^+, Fr^+, Ac^+, An^+, Car^+
6, R-1-36/2355	Sm^-, L, Vn, Sp, Spi, V, Dp^-, H1536, Hh24, Ec^-, Sub^+, Ap^+, M^+, T-D^+, Me^+, Raf^+, So^-, Du^-, Rh^+, Fr^+, Ac^+, An^+, Car^+
7, R-1-36/2535	Sm^-, L, Vn, Sp, Spi, V, Dp^{gr}, H1536, Hh24, Ec^-, Sub^-, Ap^-, M^+, T-D^+, Me^+, Raf^+, So^-, Du^-, Rh^+, Fr^+, Ac^+, An^+, Car^+
8, R-1-36/2551	Sm^-, L, Vn, Sp, Spi, V, Dp^-, H1536, Hh24, Ec^-, Sub^-, Ap^-, M^-, T-D^-, Me^+, Raf^+, So^-, Du^-, Rh^+, Fr^+, Ac^+, An^+, Car^+

Explanation; Sm^- and Sm^b: substrate mycelium colourless and dark brown, respectively; L: the habitus of aerial mycelium lanatus; Vn: spore colour venetus; Sp: sporophore spiral; Spi: spore surface spiny; V: the shape of spores variable (electron opt.); Dp^- and Dp^{gr}: soluble pigment absent and green, respectively; H1536: the titer of indirect haemagglutination 1536; Hh24: inhibition titer of haemagglutination 24; Ec^+ and Ec^-: antibiotic activity and inactivity, respectively, against *E. coli;* Sub^+ and Sub^-: inhibitory effect or its lack towards *B. subtilis* ; Ap^+ and Ap^-: antagonistic effect or its lack on other strains of the population (not on all strains !); M^+ and M^-: chromogenic and achromogenic, respectively; T-D^+ and T-D^-: Tresner-Danga reaction positive and negative, resp.; Me: mesoinosite; Raf: raffinose; So: sorbite; Du: dulcite, Rh: rhamnose; Fr: fructose; Ac: $(NH_4)_2CO_3$; An: NH_4NO_3; Car: carbamide. In case of C- and N-sources + means positive utilization, — means lack of utilization. Between strains R-1-36/2355 and R-1-36/2043 considerable difference existed in the intensity of antibiotic activity.

bility of the populations of species were taken into account at the determinations and on this basis the value of diagnostic features characteristic of the individual species were also considered. On the other hand, questions as *e.g.* the occurrence of antibiotic-producing soil microbes, might be put in an entirely new light, if the activity of populations rather than that of certain strains of a species were studied.

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UNTERSUCHUNGEN ÜBER EINE BODEN-POPULATION EINES STREPTOMYCES CHARTREUSIS ÖKOTYPS

Die Resultate der kulturellen und morphologischen Analyse einer *Str. chartreusis* Rendsina-Boden-Population — deren Stämme mit dem *Str. chartreusis* IPV 554x authenthischen Stamm auch mit serologischen Methoden identifiziert wurden — weisen auf die Labilität gewisser wichtiger und unter dem Gesichtspunkte der systematischen Determination als entscheidend betrachteter Eigenschaften hin. So ist es uns gelungen, aus einer einzigen Population eines *Str. chartreusis* Ökotyps chromogene und achromogene, antibiotisch wirksame und inaktive, gegenseitig antagonistisch wirkende usw. Stämme zu selektieren.

ИЗУЧЕНИЕ ПОЧВЕННОЙ ПОПУЛЯЦИИ ОДНОГО ЭКОТИПА ВИДА STREPTOMYCES CHARTREUSIS

Результаты культурального и морфологического анализа изолированной из рендзинной почвы популяции *Str. chartreusis*, штаммы которого были идентифицированы также и серологическими методами при помощи автентичного штамма IPV 554x *Str. chartreusis*, указывают на некоторую лабильность в отношении определенных признаков являющихся решающими с точки зрения систематического определения. Таким образом из одной единственной популяции экотипа *Str. chartreusis* удалось изолировать хромогенные и ахромогенные, антибиотически эффективные и инактивные, взаимно антагонистически действующие и другие штаммы.

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Acta Biol. Hung., **15** (1), 65—75 (1964)

ROLE OF THE ROOT SYSTEM IN THE REGULATION OF OXIDATIVE METABOLISM IN BARLEY LEAVES

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(Received January 10, 1964)

Synopsis

The O_2-uptake of detached barley leaves steadily increases. In seven days, a 60 per cent increase over the controls (leaves of intact plants) was experienced. Parallel with the enhancement of O_2-uptake in detached leaves the increased respiration became more and more resistant to malonate and NaF. Simultaneously with the increased respiratory rate in detached leaves the activity of glucose-6-P dehydrogenase also increased. The stimulation of O_2-uptake und G-6-P dehydrogenase activity was parallelled by an increased accumulation of phenolics. The breakdown of proteins, which takes place in detached barley leaves and amounted to 35 per cent in 7 days, could be counteracted by 10^{-5}M kinetin. The protein content of the kinetin-treated leaves was higher than that of the controls (intact plants). The stimulation of O_2-uptake due to detachment was markedly inhibited by kinetin. The activity of G-6-P dehydrogenase and the level of phenolics were also reduced in kinetin-treated detached leaves. It is concluded that protein breakdown, induced by the removal of the root system, is responsible for the "activation" of G-6-P dehydrogenase in detached leaves which in turn leads to a shift in respiratory pathways in favour of the hexose monophosphate shunt. The enzyme "activation" is most easily explained by the assumption of proteolytic liberation of G-6-P dehydrogenase from an inactive form.

Introduction

The removal of root system results in a rapid breakdown of leaf proteins and chlorophyll. At the same time, free amino acids, ammonia and amides accumulate [2]. The detachment of leaves also leads to a drift in respiratory rate [13]. Undoubtedly, the root has a regulatory role in leaf metabolism. The nature of this regulatory function and the mechanism of the respiratory increase in detached leaves are poorly understood. Recently, it has been shown that kinetin and some other compounds are able to "replace" the root in maintaining the normal level of some substances, including proteins and nucleic acids, in the tissues of detached leaves [10, 20, 26, 29, 33]. Also, the increase in respiratory rate, due to senescence, of excised plant organs could be inhibited by kinetin-like compounds [4, 5, 20]. However, no systematic study of the possible correlation between the altered protein and respiratory metabolism of detached leaves has been undertaken. The aim of the present work was to throw some light on this problem.

In previous investigations evidence was presented that the removal of the root system results in a profound alteration of enzyme activities in the

leaf tissues [8, 15]. Among others, the activity of the hexose monophosphate shunt dehydrogenases increases markedly in detached leaves. Therefore, a study of the possible relationship between altered enzyme activity and increased respiratory rate in detached leaves appeared warranted.

To have a deeper insight into the mechanism by which the root system regulates the oxidative metabolism of the over ground organs, the root was "replaced" by kinetin and the oxidative metabolism of kinetin-treated detached leaves was investigated.

Materials and methods

Barley seedlings (Hungarian variety MFB), grown under ordinary greenhouse conditions were used throughout the experiments. The primary leaves were detached in the two-leaf stage and placed with their bases in tap water or 10^{-5}M kinetin dissolved in tap water. The cut surface of the leaf bases was renewed every day, and the solutions were changed every second day. Samples for the assays were harvested at about 10.00 a.m.

The respiratory rate (O_2-consumption) was determined at 30°C by using standard Warburg technique. Approximately 3 × 3 mm leaf pieces were floated on 0,05 M phosphate buffer pH 4.8. Respiratory inhibitors ($2 \cdot 10^{-2}$ M malonate or 10^{-2} M NaF) were also dissolved in phosphate buffer, and the pH of the solutions was adjusted if necessary. In the experiments with respiratory inhibitors the leaf pieces were preincubated for one hour before starting the manometric assay.

Standard spectrophotometric techniques [3] and a method described by MARRÈ and SERVETTÀZ [24] were used for the determination of G-6-P dehydrogenase activity. The method of MARRÈ and SERVETTÀZ was modified: instead of triphenyl tetrazolium chloride, 2.6-dichlorophenol indophenol (DPIP) was used as hydrogen acceptor. The leaf tissues were homogenized in the cold in a tenfold amount of 0.04 M Tris buffer, pH 7.5. Centrifuged extracts were used for the spectrophotometric assays. The reduction of NADP (nicotinamide adenine dinucleotide phosphate) was measured at 340 mμ, or the reduction of DPIP was followed et 600 mμ. The two methods gave essentially identical results. The coupled reaction (DPIP reduction) proved to be more sensitive. The results obtained with the modified Marrè-Servettàz method are reported in the present paper. The reaction mixture applied was as follows: G-6-P 2.5 μmol, NADP 0.5 μmol, $MgCl_2$ 5 μmol, DPIP 0.25 μM, enzyme corresponding to 25 mg fresh weight of leaf tissue, Tris buffer pH 7.5. Final volume 2.0 ml. Light path 0.5 cm. Composition of the check: the experimental mixture minus G-6-P. It was shown in separate experiments that the plant extract contained enough endogenous diaphorase to saturate the system.

Total phenolics were determined colorimetrically in methanolic extracts (80 per cent) with the Folin-Ciocalteu reagent [32]. The results are expressed in chlorogenic acid equivalents.

Total soluble proteins were determined according to LOWRY et al. [18] in buffer extracts (Tris buffer pH 7.5). O_2-uptake, G-6-P dehydrogenase activity and phenol content were simultaneously determined.

The data presented in figures and tables are mean values from 4 to 6 consecutive experiments.

Results

Effect of detachment

1. *Respiration.* As shown in Fig. 1, the O_2-uptake of detached barley leaves steadily increases. By the end of the experiment (7th day), the increase over the controls was about 60 per cent.

To have some information on the relative importance of alternative respiratory pathways in the enhanced respiration of detached barley leaves, respiratory inhibitors (malonate, an inhibitor of the Krebs-cycle, and NaF, inhibitor of glycolysis) were applied. It may be seen from Fig. 1 that the res-

Table 1

Effect of detachment on the sensitivity of respiration to fluoride in barley leaves (Per cent inhibition of O_2-uptake by 10^{-2} M NaF)

	Days after detachment						
	1	2	3	4	5	6	7
Control	48.0	40.0	55.5	42.9	40.5	52.6	42.6
Detached leaf ..	32.9	32.5	37.8	28.9	31.2	27.0	26.4

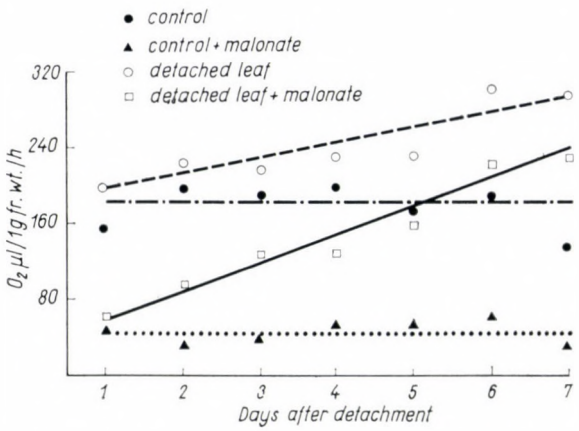

Fig. 1. Effect of detachment on malonate-sensitivity in barley leaves

piration of control leaves was strongly inhibited by malonate and that the extent of inhibition remained practically unchanged during the experiment. By contrast, during detachment the respiration of detached leaves became gradually more and more malonate resistant. The experiments with NaF showed a similar tendency, although the results were less clear-cut (Table 1). The data obtained seem to support the idea that the extra respiration of detached leaves bypasses the normal glycolytic pathway and the Krebs-cycle. A possible explanation of the malonate and NaF resistance of the stimulated O_2-uptake of detached leaves would be that the relative importance of the hexose monophosphate shunt is increased.

2. *Activity of G-6-P dehydrogenase.* The initial step of the hexose monophosphate shunt is the NADP dependent oxidation of G-6-P dehydrogenase. This step might limit the operation of the shunt [7, 22, 23]. Therefore, it was

examined whether or not there is a relationship between the development of malonate-resistant respiration and G-6-P dehydrogenase activity in detached leaves. As clearly indicated in Fig. 2, the activity of G-6-P dehydrogenase

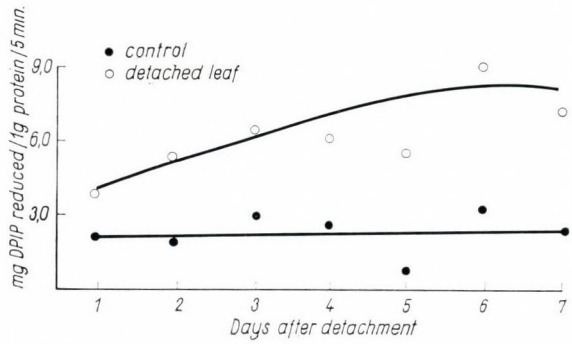

Fig. 2. Effect of detachment on the activity of glucose-6-P dehydrogenase in barley leaves

markedly increased in the detached barley leaf, and the increase parallelled the development of malonate-resistant respiration. These findings are also in line with the idea that the hexose monophosphate shunt is activated in detached leaves.

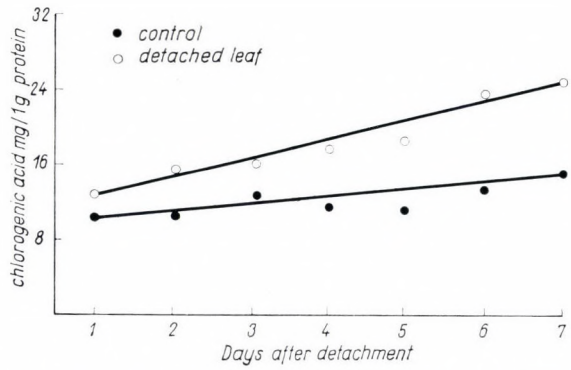

Fig. 3. Effect of detachment on the phenol content of barley leaves

3. *Changes in total phenolics.* In view of the well known connection between the operation of the hexose monophosphate shunt and aromatic biosynthesis [25], the level of phenolics was determined in detached and control barley leaves. The results are summarized in Fig. 3. It may be seen that the level of total phenolics somewhat increased with the age of the experimental plants. The increase was much more pronounced in detached leaves. The percentage stimulation of phenol accumulation in 7 days was about 70 per cent over the control.

Table 2

Effect of detachment on the activity of G-6-P dehydrogenase, level of total phenolics and respiratory rate in barley leaves (Experimental sample/control ratios)

	Days after detachment						
	1	2	3	4	5	6	7
G-6-P dehydrogenase	1.67	2.34	2.15	2.58	2.81	2.65	3.06
Total phenolics ...	1.25	1.44	1.31	1.58	1.65	1.68	1.68
Respiratory rate ..	1.18	1.11	1.17	1.21	1.36	1.63	2.22

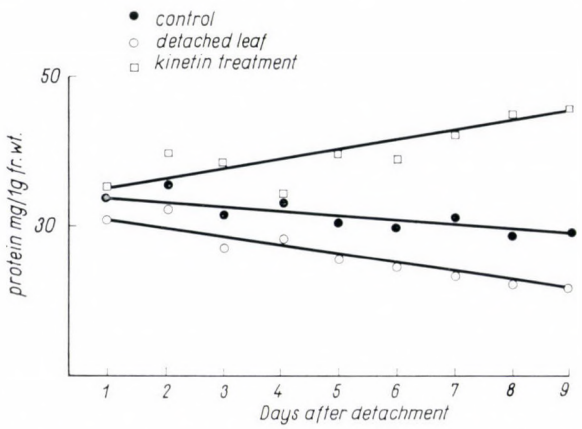

Fig. 4. Effect of detachment and kinetin treatment on the level of buffer-soluble proteins in barley leaves

In Table 2 the relative augmentation in detached leaves of respiratory rate, G-6-P dehydrogenase activity, and phenol content are shown. The tendency of the drift in these three processes being similar, a causal connection might be postulated.

Effect of kinetin

1. *Protein level.* As the normal balance of protein synthesis and breakdown in detached leaves can be maintained by kinetin treatment [10, 26,29, 33], kinetin appeared to be a useful tool in the elucidation of the problem of how far the metabolic changes discussed above are causally connected with the excessive protein hydrolysis which takes place in leaves upon the removal of the root system. As shown in Fig. 4, the barley plant is very suitable for investigations of this kind as its protein level is markedly affected both by detachment and kinetin treatment. It may be seen that the decrease in protein is considerable in the detached barley leaves and the effect of kinetin is also striking (Fig. 4).

It is remarkable that kinetin not only maintained the protein concentration at the level of the control but induced a marked protein accumulation.

2. *Respiration.* If the respiratory rise in detached leaves is causally connected with protein breakdown, kinetin must diminish the respiratory rate in

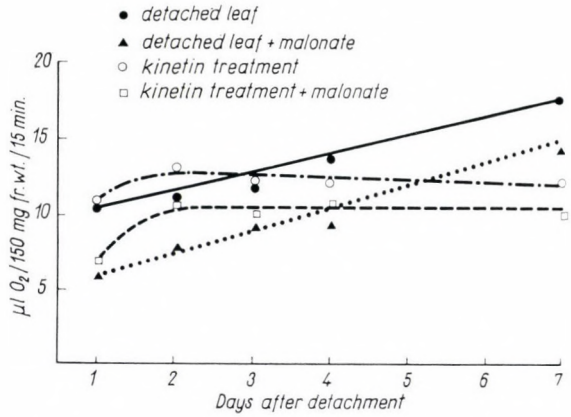

Fig. 5. Effect of kinetin on the malonate-sensitivity of respiration in detached barley leaves

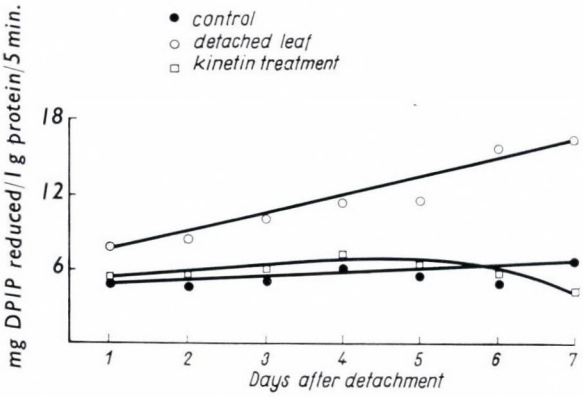

Fig. 6. Effect of kinetin on the activity of glucose-6-P dehydrogenase in detached barley leaves

excised tissues. It is clearly indicated in Fig. 5 that this is indeed the case. The O_2-uptake of detached barley leaves steadily increases (as also shown in Fig. 1) and kinetin, after a lag-period of 1 to 2 days, is able to reduce the stimulated respiration to a considerable extent. It is interesting to stress that, although the effect of kinetin on O_2-uptake is considerable, the malonate-sensitivity of detached kinetin-treated leaves is but slightly affected. There is only a slight tendency towards the restoration of the normal (high) malonate-sensitivity of O_2-uptake under the effect of kinetin treatment.

3. *The activity of G-6-P dehydrogenase.* It has been tested as to whether the kinetin-induced reduction of respiratory rise in detached barley leaves is associated with a concomitant decrease in G-6-P dehydrogenase activity. As may be seen in Fig. 6, 10^{-5} M kinetin reduces the activity of G-6-P dehydrogenase in detached leaves to the level of the controls.

4. *Phenol content.* As expected, there is a strong correlation between the kinetin-induced lowering of G-6-P dehydrogenase activity in detached leaves and their phenol level. The results of phenol determinations are summarized in Fig. 7. It may be seen that the concentration of total phenolics is decreased under the effect of kinetin treatment to or below the level of the controls.

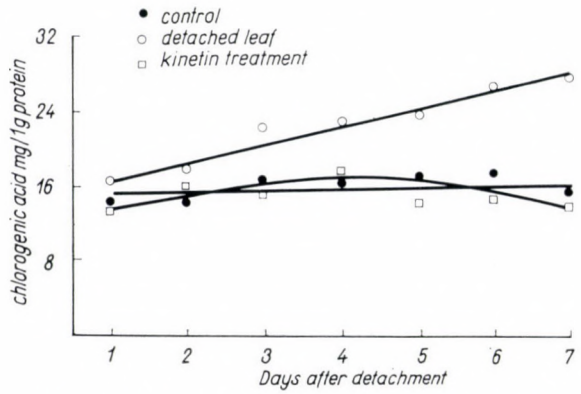

Fig. 7. Effect of kinetin on the phenol content of detached barley leaves

Discussion

Although it has been known for a long time that the removal of the root system results in the stimulation of respiratory rate in detached plant leaves [13], the biochemical mechanism responsible for the stimulated respiration remained obscure. The data presented in this paper support the view that the root system controls not only the respiratory rate of the leaves, but the relative importance of alternative respiratory pathways as well. It is suggested that in the respiration of detached leaves the participation of the hexose-monophosphate shunt increases. This is indicated by the following facts:

1. The malonate- and fluoride-sensitivity of the respiration of detached leaves decreases. This is a suggestive evidence for the increased role of a non-tricarboxylic-acid pathway, although it does not constitute a final proof, as the applied inhibitors were not fully specific [14, 36].

2. The key enzyme of the hexose-monophosphate shunt (G-6-P dehydrogenase) is activated simultaneously with the respiratory rise in detached leaves. It is noteworthy that a fairly strong correlation was found between the activa-

tion of G-6-P dehydrogenase and the *in-vivo* activation of the hexose-monophosphate shunt in diseased tissues as well [3, 17, 19, 30, 31, 34]. The same is true for the stimulated respiration of ageing (washed) storage tissue slices [1, 22, 23, 28]. Such a correlation between the activation of G-6-P dehydrogenase and activation of the hexose-monophosphate shunt seems likely in the case of detached leaves as well.

3. Simultaneously with the onset of increased respiration and G-6-P dehydrogenase activity, phenolics accumulate in the detached leaves. KIRÁLY [16] has also found an increased phenol level in detached wheat leaves. The accumulation of phenolics might be regarded as an indirect evidence for the enhanced operation of the hexose-monophosphate shunt, as at least a part of the carbon atoms of the aromatic ring derive generally from intermediates of the shunt [25]. The ubiquitous appearance of aromatic compounds in diseased plants is also regarded as being due to the activation of the hexose-monophosphate pathway [9].

The evidence is, therefore, very strong in favour of the idea that the importance of the hexose-monophosphate shunt is increased in detached leaves. The question arises: what are the biochemical factors responsible for the change? The following mechanisms might come into consideration: (i) The increase in activity of rate-limiting enzymes (G-6-P dehydrogenase, etc.). (ii) The rate of reoxidation of NADPH [7]. A comparison of the absolute values of G-6-P oxidation in cell-free extracts of barley leaves and the *in-vivo* respiratory rates indicates that G-6-P dehydrogenase might be a limiting factor of hydrogen transport in barley. Thus the activation of G-6-P dehydrogenase might have a regulatory role. Preliminary investigations show that the possibility of reoxidation of NADPH is also greater in detached leaves than in the controls [12]. In this respect, the speed of NADPH oxidation via the glutatione -ascorbic acid system [21] and the operation of a NADPH $\rightarrow$ NAD transhydrogenase [11, 27] is being considered.

Until now these two systems have been regarded as potentially the most important ones in the reoxidation of NADPH in the plant cell. However, recently evidence was presented for the direct *in vivo*- oxidation of NADPH as well which takes place without the intervention of transhydrogenase [27].

The accumulation of phenolics might also play a part in "pulling" the shunt, the biosynthesis of phenolics being also a NADPH-consuming process [25].

As to the nature of the activation of G-6-P dehydrogenase (and other enzymes) in detached leaves, we have come to the conclusion that this might be connected with the stimulation of protein breakdown. This conclusion is based on a detailed study of the behaviour of 18 enzymes in detached leaves [8, 15]. Except two (glycolic acid oxidase [6] and starch phosphorylase), all investigated enzymes were found to be activated upon detachment of leaves.

Evidence was presented that the activation was not due to protein synthesis [8, 15]. This was particularly evident with G-6-P dehydrogenase, the activity of which was dramatically reduced in detached leaves upon kinetin treatment associated with protein synthesis (Fig. 6). Studies on the behaviour of other enzymes in kinetin-treated detached leaves are in progress. It seems likely that the excessive protein breakdown liberates latent enzymes from an inactive form. The liberated enzymes apparently play an active part in the regulation of oxidative metabolism.

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DIE ROLLE DES WURZELSYSTEMS IN DER REGULIERUNG DES OXYDATIVEN STOFFWECHSELS DER GERSTENBLÄTTER

Die O_2-Aufnahme von abgetrennten Blättern der Gerstenkeimpflanzen nimmt ständig zu. Nach 6 Tagen ist die Atmungsintensität 60% höher als bei der Kontrolle (Blätter von intakten Pflanzen). Parallel zu der Atmungssteigerung der isolierten Blätter wird die O_2-Aufnahme gegen Malonsäure und NaF allmählich mehr und mehr resistent. Parallel zur Atmungssteigerung nimmt in den isolierten Blättern die Aktivität der Glucose-6-Phosphat-Dehydrogenase zu und Phenolverbindungen häufen sich an. Nach der Isolierung setzt in den Blättern ein Eiweissabbau ein, der am 7. Tage eine 35%-ige Eiweissabnahme zur Folge hat. 10^{-5} M Kinetin hemmt den Proteinabbau. Der Proteingehalt von mit Kinetin behandelten Blättern ist grösser als bei den Kontrollen (intakte Pfanzen). Die durch die Isolierung verursachte Atmungssteigerung wird durch Kinetin stark herabgesetzt. Unter dem Einfluss der Kinetinbehandlung nimmt in den abgetrennten Blättern die Aktivität der Glucose-6-Phosphat-Dehydrogenase ab und die Anhäufung von Phenolverbindungen wird gehemmt. Aus den Ergebnissen geht hervor, dass der nach Abtrennung der Blätter in Gang gesetzte Proteinabbau mit der Aktivierung von Glucose-6-Phosphat-Dehydrogenase verknüpft ist, die das relative Verhältnis der alternativen Atmungswege zugunsten der direkten Oxydation verschiebt. Die Enzymaktivierung ist wahrscheinlich durch die proteolytische Freisetzung von Glucose-6-Phosphat-Dehydrogenase aus inaktiver Form zu erklären.

РОЛЬ КОРНЕВОЙ СИСТЕМЫ В РЕГУЛИРОВАНИИ ОКИСЛИТЕЛЬНОГО ОБМЕНА ЛИСТЬЕВ ЯЧМЕНЯ

Поглощение O_2 листьями проростков ячменя с удаленной корневой системой постепенно увеличивается. Через шесть дней интенсивность дыхания превышает контроль (листья цельных растений) примерно на 60%. Параллельно повышению интенсивности дыхания изолированных листьев, поглощение O_2 становится все более устойчивым в отношении малоновой кислоты и NaF. Параллельно повышению интенсивности дыхания в изолированных листьях увеличивается активность дегидрогеназы глюкозо-6-фосфата и наблюдается накопление фенольных соединений. После изолирования в листьях начинается распад белков, в результате чего на седьмой день количество белков снижается на 35%. Кинетин в концентрации 10^{-5} М препятствует распаду белковых веществ. Содержание белка в листьях, обработанных кинетином, превышает контрольные (цельные растения). Кинетин в значительной степени снижает обусловленную изолированием стимуляцию дыхания. Под влиянием обработки кинетином в срезанных листьях снижается активность дегидрогеназы глюкозо-6-фосфата, а также и степень накопления фенольных соединений. Из полученных результатов можно сделать заключение, что распад белков, начавшийся после устранения корневой системы, связан с активированием дегидрогеназы глюкозо-6-фосфата, отодвигающей относительно соотношение альтернативных путей дыхания в сторону апотомического окисления. Активация фермента обусловлена вероятно его протеолитическим освобождением из неактивной формы глюкозо-6-фосфата.

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Acta Biol. Hung., **15** (1), 77—85 (1964)

FUNCTIONAL DIFFERENTIATION OF THE THYROID GLAND INVESTIGATED BY I^{131}-AUTORADIOGRAPHY IN DECAPITATED CHICK EMBRYOS BEARING HYPOPHYSEAL HOMOGRAFTS

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(Received January 14, 1964)

Synopsis

On the basis of autoradiographic investigations authors found a significant inhibition of thyroidal I^{131} accumulation in chick embryos following decapitation at the mesencephalic level. Subcutaneous hypophyseal grafts taken from normal donors bring the I^{131} trapping capacity of the thyroids of decapitated embryos to normal level. Similar, but less intensive effect could be demonstrated following TSH injection. It is concluded that the anterior pituitary may differentiate and produce TSH without hypothalamic connections at least in birds and in the embryonic stage. Although no I^{131} trapping capacity was present, the histological differentiation at the light microscopical level of the thyroids of decapitated embryos showed only a slight delay. The possible role of differences in the ultrastructure of thyroids is discussed.

Introduction

Previous investigations [8] revealed that the I^{131} trapping capacity of chick embryos was highly inhibited or almost completely extinquished following decapitation in early stages of embryonic development, i.e. in stages 12—13 according to HAMBURGER and HAMILTON [7].

This clearly shows that the onset of thyroidal I^{131} accumulation in chick embryos depends on pituitary thyrotropin (TSH) secretion. Other experiments [11] have shown furthermore that thyroids of normal embryos, before reaching the developmental stage in which TSH secretion starts (+37), as well as those of decapitated ones, are fairly sensitive to exogenous TSH. As the decapitated embryo has neither diencephalon, nor pituitary gland, this seemed to be an appropriate experimental model for the investigation of functional development of thyroids free from pituitary influence, on one hand, and for the study of the functional and morphological differentiation of the adenohypophysis if independent from the central nervous system on the other. Particular interest in this respect has been raised by experimental findings of HALÁSZ and associates [6] who observed that adenohypophyseal tissue would maintain its normal histological picture and its functional capacity exclusively following homotrans-

plantation in the basalventral area of the hypothalamus. This area was therefore termed by these autors as 'hypophyseotrophic area". The question remained open, however, whether or not the histological and functional differentiation of the embryonic pituitary requires the same intimate connection with the diencephalon in order to be able to produce trophic hormones, or whether this could be achieved also without diencephalic connections.

Material and methods

Our experiments were performed on 51 decapitated chick embryos at midbrain level and on 7 normal ones. Decapitation was performed on the second day of incubation i.e. developmental stages 12—13 (HAMBURGER and HAMILTON [7]. The operation was carried out under a binocular stereomicroscope under sterile conditions with the aid of the electromagnetic vibrating needle of DRURY [3].

Embryos which reached the 6th day of incubation were separated into the following groups.

Group 1. Intact controls (7 embryos).

Group 2. Decapitated controls (14 embryos).

Group 3. Decapitated embryos having received pituitary homografts from donors of the 11th day of incubation and having been sacrificed at stages 35—36 (4 embryos).

Group 4. Decapitated embryos having received pituitary homografts from donors of the 11th day of incubation and having been sacrificed at stage +37 (6 embryos).

Group 5. Decapitated embryos having received pituitary homografts from donors of the 7th day of incubation and having been sacrificed at stage +38 (9 embryos).

Group 6. Decapitated embryos having received pituitary homografts from donors of the 12th day of incubation and having been sacrificed at stage 39 (3 embryos).

Group 7. TSH (Ambinon, Organon) injection into the chorioallantoid sack of decapitated embryos.

(a) 2 USP mU on the 11th day of incubation (4 embryos).

(b) 2 USP mU daily ones between the 8th and 11th of incubation (4 embryos).

Hypophyseal homografts were transplanted under the abdominal skin between the right wing and leg on the 6th day of incubation (Fig. 2*e*).

10 μC carrier free I^{131} was injected into the chorioallantoid sack of all embryos of the different groups 24 hours before sacrification. Thyroids were dissected and fixed for autoradiography in a mixture of 8 parts of 96% alcohol and 2 parts of 40% formalin. The hypophyseal grafts were fixed in Bouin solution for histological investigations. The thyroids were embedded into polywax and serially sectioned in two parallel series. One series was prestained with Haematoxylin-Eosin, the other without staining covered with Kodak AR 10 stripping film. After 7 days of incubation autoradiographs were developed, fixed and mounted in the artificial resin of Arbocoll H [12]. Unstained autographs were quantitatively evaluated with the aid of a Zeiss fast-photometer. — Hypophyseal grafts after embedding in celloidin-paraffin and sectioning in 5 μ were stained with haematoxylin-eosin, aniline blue-azocarmin and periodic acid-Schiff (PAS) reagent.

Experimental results

The autoradiographs of the thyroids confirmed our previous data [8] according to which decapitation sharply inhibits the I^{131} trapping capacity of the thyroids (Fig. 1*b*), as compared with that of unoperated normal embryos (Fig. 1*a*). In decapitated embryos bearing hypophyseal homografts, taken from embryos of the 7th day of incubation, the I^{131} accumulation of the thyroids was as strong on the 12th day (Fig. 1*c*), as in the normal ones of the same age.

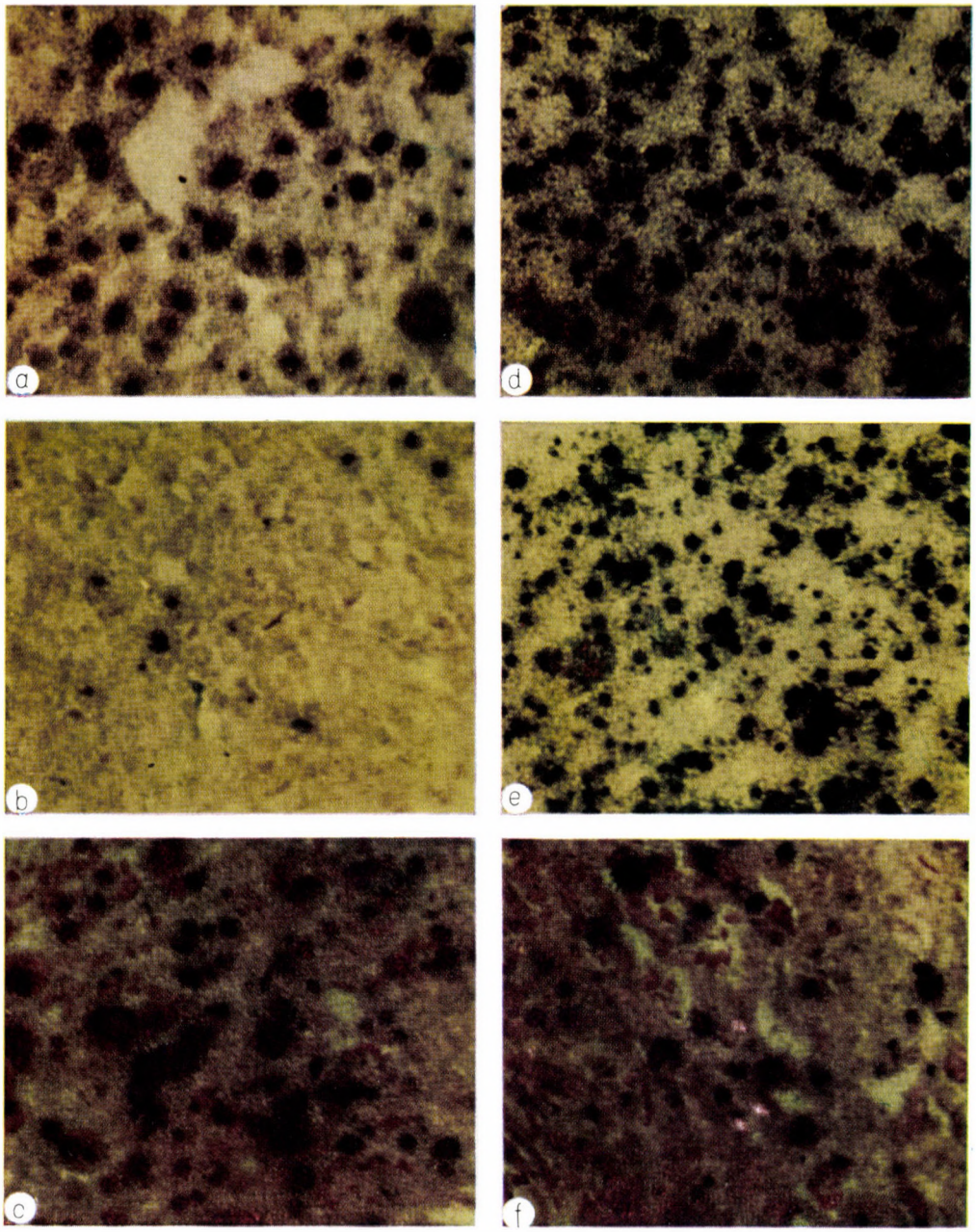

Fig. 1. Autoradiographs of the thyroids of chick embryos (Prestained with Haematoxylin-Eosin. Covered with Kodak AR 10 stripping film.). *a)* intact control (stage +38). *b)* decapitated, untreated (stage 38). *c)* decapitated having received pituitary homografts from 7 days old donors (stage +37). *d)* decapitated having received pituitary homografts from 11 days old donors (stage +37). *e)* decapitated having received pituitary homografts from 11 days old donors (stage —36). *f)* decapitated injected with 2 USP mU daily dosis of TSH from the 8th to the 11th day of incubation (stage —39)

Taking into consideration that according to our previous work [8] TSH secretion of normal chick embryos starts at the 11th day of incubation (+37 stage) — corresponding to the data of CORIN-FREDERIC and FREDERIC [2] who found the appearence of the chromophilic cells at the 9—12th days of incubation — the question arose, whether only pituitaries transplanted before the time of cytodifferentiation are able to produce TSH in the transplanted site, or whether previously differentiated pituitaries might maintain their capacity to secrete TSH after having lost their diencephalic connections in consequence of the transplantation. For the investigation of this question pituitaries of 11—12 day old embryos were transplanted into 6 days old decapitated ones. In these cases already on the 9—10th day of incubation the thyroidal I^{131} accumulation was equal to the 11th day old normal embryos (Fig. 1*e*). This result shows clearly that anterior pituitary tissue taken from donors which have already been capable of secreting TSH, if transplanted behave quite similarly to hypophyseal grafts taken from 7 days old donors. If decapitated embryos, bearing hypophyseal grafts taken either from young donors (before the onset of TSH secretion) or from older ones (with TSH secretion already started) survive the

Table 1

Experimental group	Developmental stage	Thyroidal I^{131} accumulation		Mean of thyroidal I^{131} accumulation		Significant difference between absolute values
		in absolute values	in H/D quotient	in absolute values	in H/D quotient	
Controls (7)	+38	519	5.71			against decapitated (st.+38)
	+38	590	6.60			
	—38	376	4.13			
	+38	630	6.92	564	6.20	$t = 12.96$
	38	620	6.81			$p < 0.01$
	+38	643	7.06			
	+38	571	6.27			
Decapitated (14)	—36	unmeasurably low activity				
	—36					
	—38	86		86		
	38	89				
	—38	94				
	38	73				
	+38	91		91		
	+38	91				
	+38	90				
	39	152		138		
	—39	140				
	—39	122				
	+38	573	not perfect decapitation*			
	—39	264				

*Diencephalon and hypophysis was present, only telencephalon was removed.

(Table 1, cont.)

Experimental group		Developmental stage	Thyroidal I^{131} accumulation		Mean of thyroidal I^{131} accumulation		Significant difference between absolute values
			in absolute values	in H/D quotient	in absolute values	in H/D quotient	
Decapitated bearing hypophyseal homografts (22)	11 days old hypophyses (4)	35	245	2.85*	419	4.86	against decapitated (st.—38) $t = 7.2$ $p < 0.01$
		—36	493	5.73			
		—36	390	4.76			
		—36	550	6.39			
	11 days old hypophyses (6)	+37	403	4.68	442	5.14	against decapitated (st.—38) $t = 10.5$ $p < 0.01$
		37	359	4.17			
		+37	392	4.55			
		+37	504	5.85			
		+37	504	5.85			
		+37	487	5.66			
	7 days old hypophyses (9)	38	620	6.81	590	6.48	against decapitated (st. + 38) $t = 12.4$ $p < 0.01$ against contr. $t = 0.46$ $p = 0.62$
		+38	783	8.60			
		+38	503	5.52			
		+38	532	5.86			
		+38	629	6.91			
		+38	631	6.92			
		38	320	3.51			
		38	641	6.94			
		+38	654	7.19			
	12 days old hypophyses (3)	39	982	7.11	968	7.01	against decapitated (st.—39) $t = 43.2$ $p < 0.01$
		—39	935	6.77			
		39	987	7.12			
Decapitated	+TSH treatment 1x (4)	—39	260	1.89	267	1.93	against decapitated (st.—39) $t = 9.75$ $p < 0.01$ against contr. $t = 6.08$ $p < 0.01$
		+38	293	2.12			
		—39	250	1.82			
		39	265	1.92			
Decapitated	+TSH treatment 4x (4)	—39	292	2.19	283	2.05	against decapitated (st.—39) $t = 11.7$ $p < 0.01$ against contr. $t = 5.92$ $p < 0.01$
		39	266	1.91			
		—39	301	2.21			
		—39	274	1.93			

*H/D quotient at this subgroup is calculated instead of their own decapitate d cases from the data of decapitated group in stage —38.

12th day of incubation (stages +38, —39), the I^{131} trapping capacity of the thyroid gland increases to the level found in unoperated normals (Fig. 1*d*). If instead of hypophyseal homotransplantation TSH is injected (2 USP mU y/embryo), the iodide uptake increases as compared with the untreated decapitated embryos (Fig. 1*f*), but does not reach the degree, seen in the cases bearing hypophyseal homografts.

Photometric evaluation of the unstained thyroid autographs gives similar results. The data are summarized in Table 1. The figures of the 2nd and 4th columns represent the light absorption of the autographs in $^0/_{00}$, whereas those

of the 3th and 5th columns are the so called H/D quotients. In these quotients H indicates the degree of darkening (light absorption) in the different pituitary grafted or TSH-treated groups, while D the degree of darkening in decapitated control embryos.

It appears clearly from Table 1 that in cases of hypophyseal homografts taken from donors before or after onset of TSH secretion occurring in the — 37 developmental stage of HAMBURGER and HAMILTON [7], the thyroidal I^{131} accumulation is enhanced in both groups in a statistically highly significant manner. No significant difference could be observed between the iodide uptake of normal untreated embryos and of decapitated ones bearing pituitary grafts. This implies that the transplanted embryonic pituitary has the same thyrotrophic capacity as the hypophysis in its normal site and in connection with the hypothalamus. Exogenous TSH injected either administered once only, or daily during 4 days, produced a significantly higher thyroidal I^{131} accumulation than of untreated decapitated embryos, but a significantly lower accumulation than in decapitated ones bearing hypophyseal grafts or in untreated normal controls.

The histological character of the thyroids in decapitated embryos bearing hypophyseal grafts is quite similar to that of normal ones. A slight delay in the differentiation of the thyroid gland is visible in the blank decapitated groups against unoperated embryos, which has already been described in more detail in a previous paper [8]. The formation of thyroidal follicles occurs in decapitated embryos in normal manner, but no colloid appears in them, which is in good accordance with the impairment of I^{131} accumulation.

Pituitary grafts were well vascularized, surrounded with a thin layer of connective tissue (Fig. 2*e*). The histological character and the degree of morphological development also corresponds to those in the intact controls. Fig. 2*a* shows a 12 day old normal pituitary, whereas Fig. 2*b* a transplanted pituitary exactly at the same age. Fig 2*c* normal, 17 days old adenohypophysis, while Fig. 2*d* a hypophyseal homograft transplanted on the 11th day of incubation, so that the graft was 17 day old at the time of sacrification. This gland shows a ripe fully developed picture of a normal pituitary. Positive PAS-reaction is also seen in some cells of the grafts, unfortunately, however, the PAS staining is too pale for photographic demonstration, both in the grafts and in the pituitaries of the intact controls.

Discussion

Experiments described above show that the embryonic pituitary tissue, at least in birds, is able to achieve a fairly normal level of morphological differentiation and to produce and mobilise trophic hormones without any contact with the diencephalon. This functional capacity of the grafts is independent

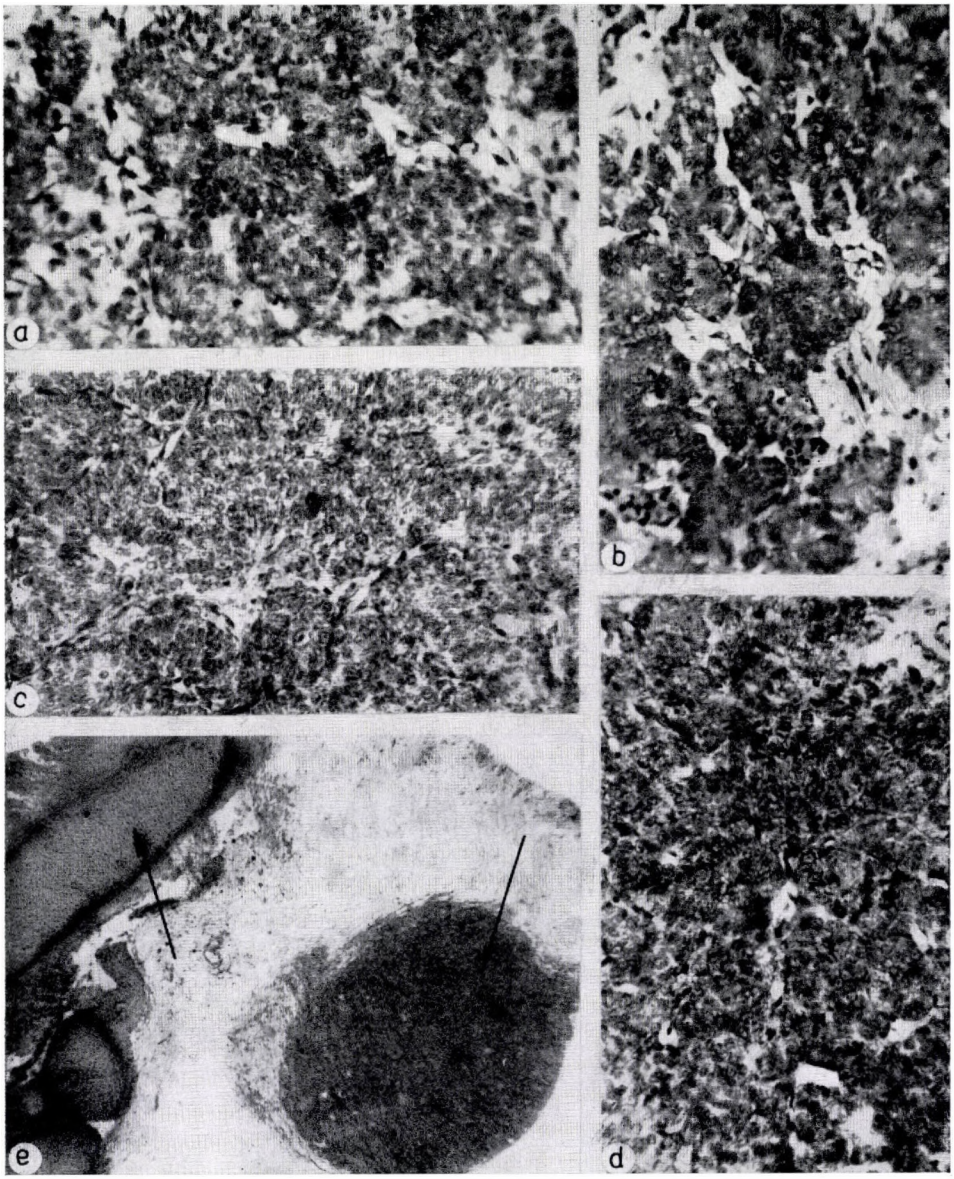

Fig. 2. Histological appearence of hypophyseal homografts (Haematoxylin-Eosin). *a)* adenohypophysis from a normal 13 days old in situ pituitary. *b)* adenohypophysis transplanted in the 6th day, from a 7 days old donor, and sacrificed on the 13th day of incubation. *c)* adenohypophysis from a normal 17 days old in situ pituitary. *d)* adenohypophysis transplanted from a donor on the 11th day of incubation into a decapitated acceptor on the 6th day of incubation. Sacrificed when the acceptor was 12 days old and the graft 17 days old. *e)* localization and histological appearence of a graft. → indicates the hypophyseal graft. ⇢ indicates the costal cartilage

from the time of transplantation, i.e. whether it takes place before or after appearence of the chromophilic cells. This latter observation raises a series of new important questions. The first question is: how long does the transplanted hypophysis maintain its function? One possibility were that TSH release from the graft during a process of necrobiotic catabolism is responsible for a transitory thyroid stimulating effect. This latter assumption appears to be in contradiction to the correct histological aspect of the pituitary grafts (Fig. 2). Pituitaries transplanted from donors of the 7th day of incubation have no TSH secretion at transplantation, so that TSH production necessarily starts only after transplantation, a fact speaking sharply against this assumption. No answer could be given to the question how long the hypophyseal transplants can maintain their function, as decapitated embryos never survive the day of hatching. We shall try, therefore, to approach this problem in another way.

From the theoretical point of view there is an other interesting problem. Is there real essential discrepancy between these experiments and the data of HALÁSZ and associates [6]? The question could be answered negatively. Many authors [4, 9, 10] stated that pituitary of newborn rats transplanted into the anterior ocular chamber has a considerable TSH activity. HALÁSZ himself (personal communication) found a fairly normal T/S ratio in cases of pituitary grafts transplanted under the renal capsule of hypophysectomized hosts. The observation of BIRESSI and coworkers [1] have confirmed these results in human anencephals. They were able to demonstrate TSH as well as ACTH in the adenohypophyses of anencephals having only lower brainstems. From these data it seems to be probable that the localization of the grafts in the "hypophysiotrophic area" of the hypothalamus may be necessary only for the histological and histochemical integrity of the grafted pituitary, but not for the TSH-secreting capacity. Another possible explanation might be the difference in the age of the hypophyseal grafts at the time of transplantation. We have transplanted embryonic pituitary tissue, while HALÁSZ and associates [6] pituitaries from 30 day old rats. GLYDON [5] describes the portal circulation of the rats hypophysis to develop only around the 5th day after birth. So transplantation of the pituitary gland might have occurred in our cases before the development of the portal circulation.

Several explanations might be given for the finding that purified TSH preparation (Ambinon, Organon) produced a markedly lower effect than did hypophyseal transplants. The dosis given might have been too low, or resorption might not have been sufficient in the embryos. First of all, however, we have to think of the possibility that exogenous TSH injected only daily could not maintain such a steady TSH level as did endogenous TSH, secreted by the hypophyseal graft. The character of the thyroidal effect, produced by TSH injection being principally the same as in cases with pituitary graft and only the degree of the effect being different, the conclusion might be drawn that

the TSH output by the pituitary graft may be at considerable height and fairly constant level.

Our experiments show, on the other hand, that thyroid gland may reach a certain degree of morphological development without being stimulated by TSH these thyroids, however, are unable to trap iodide. 24 hours following a single TSH injection the functional capacity of thyroids of decapitated embryos are normalized. As the gross anatomy and the light microscopical picture of thyroids of decapitated embryos do not show very considerable differences against those of normal ones, it is more likely that the difference might be in the fine structure of the thyroid epithelial cells. This difference in the fine structure of the secreting apparatus might be reorganized or built up very rapidly (probably in 24 hours) under TSH influence. This last interesting question remains to be elucidated with electronmicroscopical studies of similar material.

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DIE FUNKTIONELLE DIFFERENTIATION DER SCHILDDRÜSE IN DEKAPITIERTEN UND MIT HYPOPHYSENVORDERLAPPEN HOMOTRANSPLANTATEN VERSEHENEN HÜHNEREMBRYONEN MITTELS I^{131} AUTORADIOGRAPHIE

Hühnerembryonen in der Höhe des Mesencephalon dekapitiert, zeigten eine hochgradige Herabsetzung der I^{131} Aufnahme. Die I^{131} Aufnahme dekapitierter Embryonen wird durch von normalen Embryonen stammenden Hypophysenvorderlappen normalisiert. Ähnliche, nur etwas geringere Effekte sind durch von aussen eingeführtes thyreotrophes Hormon (TSH) herbeizuführen. Aus den vorgelegten Experimenten wird die Schlussfolgerung gezogen, dass

Hypophysenvorderlappen wenigstens beim Vogelembryo die Fähigkeit hat, sich auch ohne hypothalamische Steuerung zu differenzieren und TSH zu produzieren. Andererseits ist die allgemeine lichtmikroskopische Struktur der Schilddrüse dekapitierter und normaler Embryonen ziemlich ähnlich, scheint jedoch die Fähigkeit zu haben, I^{131} zu speichern. Die Rolle etwaiger lichtmikroskopisch nicht sichtbarer Unterschiede der Feinstruktur werden kurz besprochen.

АВТОРАДИОГРАФИЧЕСКОЕ ИССЛЕДОВАНИЕ ФУНКЦИОНАЛЬНОЙ ДИФФЕРЕНЦИАЦИИ ЩИТОВИДНОЙ ЖЕЛЕЗЫ ПРИ ПОМОЩИ ВКЛЮЧЕНИЯ I^{131} У ДЕКАПИТИРОВАННЫХ КУРИННЫХ ЭМБРИОНОВ В СЛУЧАЕ ГОМОТРАНСПЛАНТАЦИИ ГИПОФИЗА

Авторадиографическое исследование показало значительное снижение включения I^{131} в щитовидную железу куриного эмбриона после его обезглавливания на уровне межуточного мозга. Подкожная пересадка гипофиза от нормального животного привела включение I^{131} в щитовидную железу декапитированного эмбриона к нормальному уровню. Подобный, но менее интенсивный эффект оказала инъекция тиреостимулирующего гормона. Сделан вывод, что передняя доля гипофиза может дифференцироваться и продуцировать тирео-стимулирующий гормон не имея связи с гипоталамусом в эмбриональном возрасте у некоторых птиц. Хотя включение I^{131} не наблюдалось, тканевая дифференциация показала только небольшое изменение при обычном микроскопическом исследовании. Дискутируется возможное изменение в ультраструктуре щитовидной железы.

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Acta Biol. Hung., **15** (1), 87—94 (1964)

THE CHROMOSOMES OF CATINELLA VERMETA (MOLLUSCA, EUTHYNEURA, SUCCINEIDAE)[1]

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(Received March 25, 1964)

Synopsis

Catinella vermeta (SAY) is a common and wide spread succineid snail of North America. The genus to which *C. vermeta* belongs is considered one of the most primitive of its family because the terminal male reproductive organs lack a penis sheath. The haploid chromosome number of *C. vermeta* is 6, the diploid number is 12. These are the lowest chromosome numbers so far reliably reported for a mollusk. Chromosome numbers reported for species of other more advanced genera of the Succineidae are n = 17, 20, 21 and 22.

Within its family, *C. vermeta*'s low chromosome number and primitive position strengthens the concept that chromosome evolution in the Euthyneura has usually involved an increase in chromosomes. However, since *C. vermeta*'s chromosome number is lower than that yet found in the presumably more primitive Basommatophora and "opisthobranch" orders, the value of chromosome numbers is lessened in assessing the primitiviness or specialization of higher taxonomic groups.

Introduction

Haploid chromosome numbers reported for the molluscan subclass Euthyneura range from 12 to 72. This wide range of numbers is reduced to 12—34 by omitting the obviously polyploid numbers found in the Ancylidae [9] and Planorbidae [4, 6, 8]. The mostly aquatic Euthyneura (orders Nudibranchia, Soleolifera, Cephalaspidea, Sacoglossa, Anaspidea and Basommatophora) have 19 pairs of chromosomes or less [5] and the land species (Order Stylommatophora) have 21 or more pairs [20]. The only known exception to this is in the Stylommatophoran family Succineidae.

Chromosome studies on members of the land snail family Succineidae are restricted to six papers [14, 16, 18, 22, 31, 32]. The chromosome numbers reported in these papers range from 17 in the three *Succinea* species studied from Japan, to 22 for *Succinea putris* in Europe (Table 1). These numbers span the gap between the lower Euthyneura and the more advanced species belonging to the Stylommatophoran orders Orthurethra, Mesurethra and Sigmurethra. (The range of chromosome numbers reliably reported for the Orthrurethra are

[1] This investigation was supported (in part) by a Public Health Service research career program award (number 1-K3-AI-19, 451—01) and by research grants 2 T1 AI 41—06A1 from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service and GB-787 from the National Science Foundation, Washington, D.C., U.S.A.

n = 24—28 [11]; for the Mesurethra they are n = 24—27 [12]; and for the Sigmurethra they are n = 21—34 [20].)

In view of the much higher chromosome numbers so far known to occur in euthyneuran snails, it was indeed a surprise to find a succineid land snail that had only 6 pairs of chromosomes. It is on this species, *Catinella vermeta* (SAY), that this paper is based.

Table 1

Chromosome numbers reported in the Succineidae

Species	Chromosome Number		Source	Reference
	n	2n		
Catinella				
C. vermeta[1]	6	12	Michigan, U.S.A.	This report
Succinea				
S. hirasei[2]	17	34	Japan	[22]
S. horticola[2]	17	34	Japan	[18]
S. kwansae[2]	17	34	Japan	[22]
S. ovalis[3]	20	40	N.J., Maine, U.S.A.	[14]
S. olvalis[3]	21	—	Virginia, U.S.A	[16]
S. putris[4]	22	—	Europe	[31, 32]

[1] Subgenus *Mediappendix*.
[2] I cannot determine from the literature to which subgenus the three Japanese species belong.
[3] Subgenus *Novisuccinea*.
[4] Subgenus *Succinea* s. s.

Systematics

According to the classification of ODHNER [30], the genus *Catinella* is divided into three subgenera, *Catinella* s.s. (restricted to the Pacific Islands), *Quickella* (distributed on the Atlantic and Channel coastal regions of Europe), and *Mediappendix* (distributed in North America). ZILCH [40] follows this same classification. *Catinella vermeta* (SAY) belongs to the latter subgenus, which includes 10 other species, listed in Table 2.

Catinella vermeta is the first species from North America named in the genus as now understood. *C. vermeta* also has the widest distribution, ranging from New Jersey west to Michigan in the north and from North Carolina to Oklahoma in the south.

In previous literature *Catinella vermeta* commonly has been known also as *Quickella vagans* (PILSBRY) [27, 37]. However, I am following the nomenclature of HUBRICHT [15] who found that the type lot of *Succinea campestris*

Table 2

Species of Catinella *occurring in continental U.S.A. and their presently understood distribution*

Species	Original generic designation	Author	Type Locality[1]	Distribution by state
C. gelida	*Succinea*	F. C. BAKER, 1927 [1]	Boone Co., Ill.	Illinois
C. hubrichti	*Catinella*	GRIMM, 1960 [13]	Worcester Co., Md.	Maryland
C. oklahomarum	*Quickella*	WEBB, 1953 [39]	Pushmataha Co., Okla.	Oklahoma
C. oregonensis	*Succinea*	LEA, 1841 [24]	"Oregon"	Mont., Idaho, Wash., Ore., Calif.
C. pinicola	*Catinella*	GRIMM, 1960 [13]	Worcester Co., Md.	Maryland
C. pugilator	*Catinella*	HUBRICHT, 1961, [15]	Currituck Co., N.C.	N. Carolina
C. rehderi	*Oxyloma*	PILSBRY,1948 [37]	Lincoln Co., Wash.	Wash., Ore.
C. stretchiana	*Succinea*	BLAND, 1865 [3]	Washoe Co., Nev.	Wyo., Idaho, Utah, Nev., Calif.
C. texana	*Catinella*	HUBRICHT, 1961 [15]	Milan Co., Tex.	Texas
C. vermeta	*Succinea*	SAY, 1824 [38]	Posey Co., Ind.	N. J., Md., N. C., Ohio, W. Va., Ky., Tenn., Mich., Ind., Okla.
C. wandae	*Quickella*	WEBB, 1953 [39]	Wabaunsee Co., Kan.	Kan., Wyo.

[1] County and state only are given.

vagans (PILSBRY) [35] contained a mixture of two species which, on shell characters, could not be distinguished from *S. campestris* SAY and *C. vermeta* (SAY). The original description of *S. vagans* was based on the shells of *S. campestris*.

Materials and methods

Specimens used in this study were obtained from 2 localities in Michigan, U.S.A.: Raisin River about 3 miles south of Tecumseh, Lenawee County; and Raisin River, 1 mile north of Clinton, Washtenaw County. The tissues were killed, fixed and preserved in NEWCOMER's fluid [29] and stained by the acetic-orcein squash technique [23]. Observations were made with Nikon (Nippon Kogaku) microscopes using 100× (n.a. 1.25) oil immersion objectives and 10× and 30× oculars. The chromosomes were drawn with the aid of a camera lucida and reproduced at a table top magnification of 5700×. Photographs were taken using a 10× ocular oil immersion objective, a Kodak Wratten 57A (green) filter, and Kodak High Contrast Copy film.

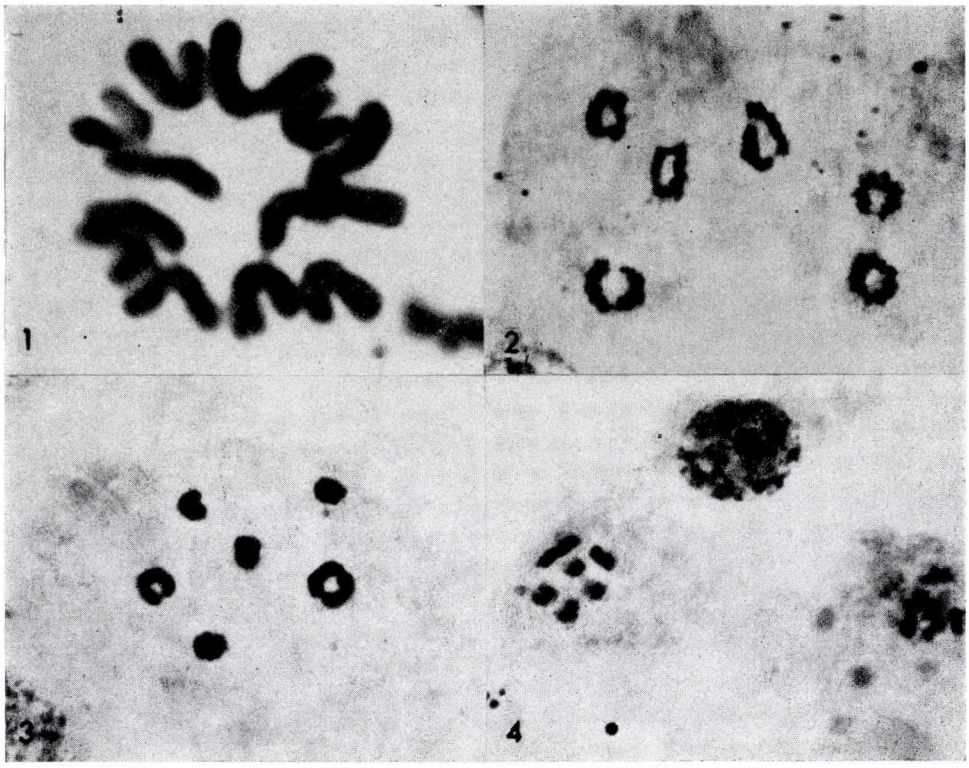

Fig. 1. Spermatogonial metaphase chromosomes (× 4750). A camera lucida drawing of these chromosomes is shown in Fig. 6. — *Fig. 2.* Diakinesis bivalents (♂) (× 1780) — *Fig. 3.* Metaphase I bivalents (♂) (× 1780). A camera lucida drawing of these chromosomes is shown in Fig. 5. — *Fig. 4.* Telophase II monads (♂) (× 1780)

Observations

The diploid chromosome number of *Catinella vermeta* is 12. The 6 pairs of chromosomes can be discerned quite readily in the spermatogonial cells (Figs. 1 and 6). At least 1 pair of chromosomes (perhaps 2) is submedianly constricted, the others are metacentrics. Both the largest and smallest pairs are quite plainly medianly constricted. There is a secondary constriction near the terminal end of an arm of one of the two largest chromosomes. This may be the nucleolar organizer. The largest chromosome measured was 5.6 micra long, the smallest was 3.0 micra long.

At early diakinesis the chromosomes appear as circles of varying sizes (Fig. 2). Presumably each of these bivalents has two chiasmata. Sometimes one of the diakinesis bivalents appears as though it has only one chiasma, *i.e.*, it does not appear as a circle. Metaphase I bivalents appear as smaller more compact circles, and here again one bivalent often does not form a com-

plete circle (Figs. 3 and 5). Metaphase I bivalents measure 2.3 to 3.0 micra long in greatest dimension. At Metaphase II the dyads appear as "dumb bells". or paired "dumb bells", measuring 2.1 to 2.6 micra in length.

Discussion

The chromosome numbers of *Catinella vermeta* (n = 6, 2n = 12) are the lowest numbers reliably reported for a mollusk. A haploid number of 6 was reported [26] for the aquatic basommatophoran *Physa gyrina*, but this report

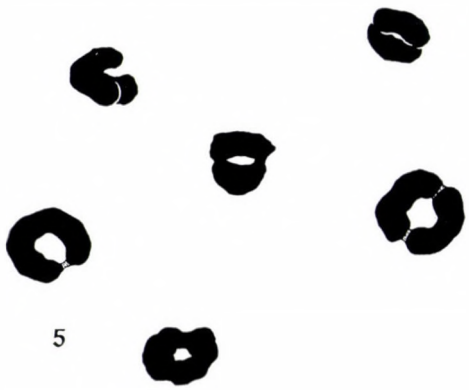

Fig. 5. Camera lucida drawing of Metaphase I bivalents (♂). A photograph of these chromosomes is shown in Fig. 3. — *Fig. 6.* Camera lucida drawing of spermatogonial metaphase chromosomes. A photograph of these chromosomes is shown in Fig. 1

has been shown to be in error [10]. The number n = 6 was reported for the cephalopod *Sepia officinalis* [25], but this report is also not reliable [19].

The low chromosome number of *Catinella vermeta* is of considerable interest in regard to current concepts of chromosomal evolution in mollusks. When chromosomal change in euthyneuran snails follows known phylogenetic advancement it seems nearly always to do so with an increase in the direction of greater specialization [11, 16, 17, 20]. If this generally holds true, then this increase of chromosome number with specialization, coupled with the conservativeness of chromosome numbers that is usually observed in snails, can shed light on the relative degree of specialization in groups of doubtful phylogenetic placement (providing the groups in question exhibit different chromosome numbers) [7].

On morphological grounds (on the basis of the internal structure and arrangement of the kidney and ureter) the stylommatophoran suborder Heterurethra, to which *Catinella* and the other succineids belong, is thought to be

somewhat intermediate in specialization between the primitive Orthurethra and Mesurethra on the one hand, and the more highly advanced Sigmurethra on the other hand [2, 33, 34].

The ranges of chromosome numbers reliably reported for the stylommatophoran suborders are as follows: Orthurethra, n = 24—28; Mesurethra, n = 24—27; Heterurethra, n = 6—22; and Sigmurethra, n = 21—34. Because of the low chromosome numbers of the Heterurethra, it has been suggested that this suborder is perhaps more primitive than the Orthurethra and Mesurethra [11]. Moreover, because the Japanese succineids had a smaller number of chromosomes than that commonly found in the Basommatophora (from which the Stylommatophora are generally thought to be derived), it was thought that perhaps the Heterurethra were not derived from a basommatophoran ancestor, but more directly from an opisthobranch [11]. It is interesting to note in this regard that Dr. JOYCE E. RIGBY (personal communication) of Queen Elizabeth College, London, considers Succinidae to be "opisthobranchs", rather than stylommatophorans, on the basis of the functional morphology of their alimentary canals. Reliable reports of chromosome numbers in the various opisthobranch orders are as follows: Notaspidea, n = 12 (1 species); Nudibranchia, n = 13 (13 species); Soleolifera, n = 16 (1 species); Cephalaspidea, n = 17 (2 species); Sacoglossa, n = 17 (1 species); Anaspidea, n = 17 (2 species) [21, 28].

Since there are no euthyneuran snails with chromosome numbers nearly as low as that found in *Catinella vermeta*, then its chromosome number *per se* does not seem to give an indication of its relationship to other groups. Since *Catinella* is not considered more primitive than the opisthobranchs for which information on chromosome numbers is available, its very low chromosome number would tend to suggest that chromosome numbers are less reliable characters as indicators of relative degree of primitiveness or specialization than previously thought, at least between higher taxons. However, chromosome data on snails are still so fragmentary that it is too early to completely disregard the evidence which supports the idea that decrease in chromosome numbers in the Euthyneura is rare, and that chromosomal change (in number) in this group is usually an increase which follows specialization or phylogenetic advancement. In addition, within the family Succineidae, it is interesting to note that on details of anatomy *Catinella* is considered more primitive than the other genera [30]. In view of this, the reader is again reminded that *Catinella* has the lower chromosome number.

Acknowledgements

Grateful acknowledgement is made to C.M. PATTERSON for help in securing the specimens on which this paper is based and for technical assistance in making the slides; to J. M. HUBER for help in preparing the manuscript; and to HENRY van der SCHALIE for facilities and many kindnesses.

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DIE CHROMOSOMEN VON CATINELLA VERMETA (MOLLUSCA, EUTHYNEURA SUCCINEIDAE)

Catinella vermeta (SAY) ist eine gewöhnliche und verbreitete Succineide von Nord-Amerika. Die *C. vermeta* enthaltende Gattung ist als die primitivste ihrer Familie anzusehen, da keine Penisscheide in den terminalen männlichen Geschlechtsorganen vorhanden ist. Die haploide Chromosomenzahl von *C. vermeta* ist 6, die diploide 12. Sie sind die niedrigsten Chromosomenzahlen, die bisher sicher für eine Molluske berichtet wurden. Für Arten der fortgeschritteneren Genera der Succineiden wurden Chromosomenzahlen von n = 17, 20, 21 und 22 mitgeteilt. Die niedere Chromosomenzahl und primitive Stellung von *C. vermeta* bestätigt innerhalb der Familie Succineidae die Auffassung, dass eine Erhöhung der Chromosomenzahl in der chromosomalen Evolution der Euthyneure allgemein miteinbezogen wurde. Dass aber in *C. vermeta* geringere Chromosomenzahl gefunden wurde als in den wahrscheinlich primitiveren Basommatophora und "opistobranchen" Ordnungen, deutet darauf hin, dass die Chromosomenzahlen keinen allzu grossen Wert in der Beurteilung von Primitivität oder Spezialisierung von höheren taxonomischen Gruppen haben.

ХРОМОСОМЫ CATINELLA VERMETA (MOLLUSCA, EUTHYNEURA, SUCCINEIDAE)

*Catinella vermeta (*SAY*)* является обыкновенной и распространенной сукцинеидной улиткой Северной Америки. Род содержащий вид *C. vermeta* принимается как самый первобытный этого семейства, ввиду отсутствия влагалища пениса из терминальных мужских половых органов. У *C. vermeta* гаплоидное число хромосом равняется 6, диплоидное же 12. Эти числа являются самыми низкими, достоверно сообщенными о моллюсках. Число хромосом у более развитых родов Succineidae равняется n= 17, 20, 21 и 22.

Низкое число хромосом и первобытное положение *C. vermeta* внутри семейства Succineidae подтверждают мнение, что эволюция хромосом у *C. vermeta* происходит путем повышения числа хромосом. Число хромосом у *C. vermeta* ниже чем у до сих пор изученных представителей Basommatophora и опистобранхных отрядов, которые вероятно представляют собой более первобытные группы. Это обстоятельство снижает значение чисел хромосом в оценке первобытности или специализации высших таксономических групп.

Acta Biol. Hung., **15** (1), 95—117 (1964)

THE "CROSSING OVER" SYNAPSE: AN ELECTRON MICROSCOPE STUDY OF THE MOLECULAR LAYER IN THE CEREBELLAR CORTEX

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(Received March 25, 1964)

Synopsis

The ultrastructure of the axon-spine synapses in the cerebellar cortex molecular layer is studied from the viewpoint of the general principles of synaptic arrangement between neurons. The dominant axon-spine synapses of this region are considered as a characteristic example of synaptic linkage by "*crossing-over*" contacts, a device to secure maximum divergence and convergence with a minimum of complexity (and irregularity) in spatial arrangement of the nerve elements involved. An attempt is made to identify under the electron microscope the smaller dendritic branches of Purkinje, basket- and outer star neurons. This leads up to a better insight into the probable conditions under which these neurons might be brought to action, and to some understanding of their possible role in the transmission and processing of impulse patterns in the cerebellar cortex. Particular attention is paid to the unusually close relations between glial and nervous tissue in this region, with some speculations on their possible significance and on differences in architecture of the grey matter in general.

Introduction

Previous electron microscope studies by GRAY [6] of the cerebellar cortex molecular layer have shown that the synaptic relations between the parallel fiber (granule axon) system and Purkinje neuron dendrites is essentially as deduced on the basis of Golgi preparations. They have, moreover, substantiated the views of FOX and BARNARD [3] on the dendritic spines as the main postsynaptic elements of Purkinje dendrites. There are, however, some problems that need further elucidation as *e.g.* (i) the question whether the presynaptic sites of the parallel fibers are short side branches with bulbous terminal enlargements or simply fusiform thickenings of the axons and (ii) the identification of the dendrites of other — basket —, outer star and Golgi-neurons under the electron microscope. This would be particularly rewarding from the viewpoint of getting some better insight into the anatomical possibilities of synaptic stimulations of these neurons. These investigations have been started and conducted primarily with this latter (ii) question in view, as from a series of recent investigations into the neuronal connexions of the cerebellar cortex [3, 9, 12, 13, 14, 17] the mode of neuron coupling in the cerebellar cortex has been brought once again into the focus of interest and most recent physiological findings [1] have proved this interest to be highly justified.

Material and methods

The investigations were made on the cerebellar cortex of the cat — mainly upper vermis — fixed according to GRAY [4] in buffered osmic acid, embedded in Epon-resin and cut into ultrathin sections on the LKB ultrotome. The sections were mounted on grids (300 mesh) without membranes and afterstained with lead hydroxide. They were investigated with the aid of the table electron microscope "Tesla" (Brno, CSSR).

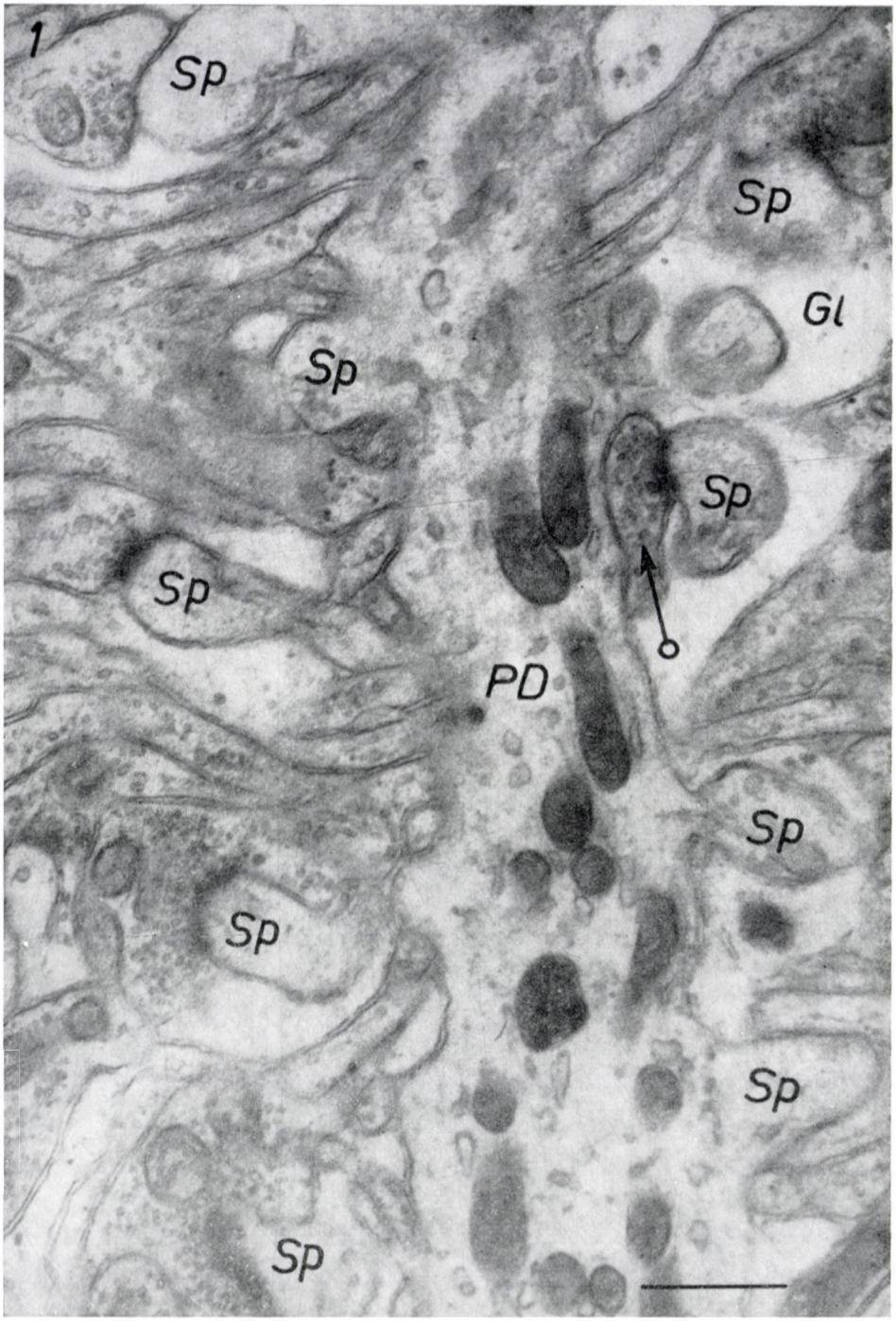
Results

Purkinje neuron dendrites and their synapses

The main dendrites of the Purkinje neurons can be identified by tracing them upwards from the cell bodies. They are large dendrites filled with an unusually strong tubular system and a strange endoplasmic membrane system consisting of groups of sacs tightly pressed together and flattened towards each other. They are almost completely sealed from their environment by an envelope of glial tissue, which only occasionally admits an axon terminal to establish a synaptic contact — generally of GRAY's [5] type 2 — with the dendrite. The main dendrites and their synapses, however, are beyond the scope of this paper and shall be described in detail elsewhere. They have been used here only to identify the smaller tertiary dendrites — the so called "spiny branchlets" — that originate from them. Such "spiny branchlets" are shown in Figs. 1 and 2 in longitudinal and in Figs. 3 and 4 in transversal section. They have a characteristic "opaque" plasma structure, dark elongated mitochondria and spheroid or irregular endoplasmic sacs loosely scattered and connected occasionally among each other by relatively wide tubuli, which have no resemblance or connexion whatever with the tubular system of the main dendrites. Most remarkable, however, are their short relatively thick spines very regularly spaced (Figs. 1 and 2) and giving an asteroid profile in transverse section. The spines are always devoid of mitochondria, have the same opaque structure as the tertiary dendrites and contain one or few tubular elongations of the endoplasmic sacs of the dendrites which in the terminal parts of the spines widen up to occupy a large space (Figs. 2, 5, 9 and 10). Three important questions have to be considered in connection with the Purkinje dendrite spines, viz.: (i) their relation to the parallel fibers, (ii) their secondary branches, and (iii) their relation to the glial tissue.

(i) *The parallel fiber — Purkinje spine synapse* is established in most cases by simple apposition of the somewhat thicker end of the spine to a fusi-

Fig. 1. Longitudinally cut Purkinje neuron dendritic branch *(PD)* with characteristic regular arrangement of short relatively thick spines *(Sp)* which in their initial part are surrounded by light glial *(Gl)* plasma and which establish synaptic contacts with the transversally running parallel fibers. Ringed arrow points to axon profile with synaptic vesicles having an immediate unspecific contact with the surface of the dendrite, but specialized synaptic attachment only with reclining spine. If not specially labelled scale represents in all cases 1 micron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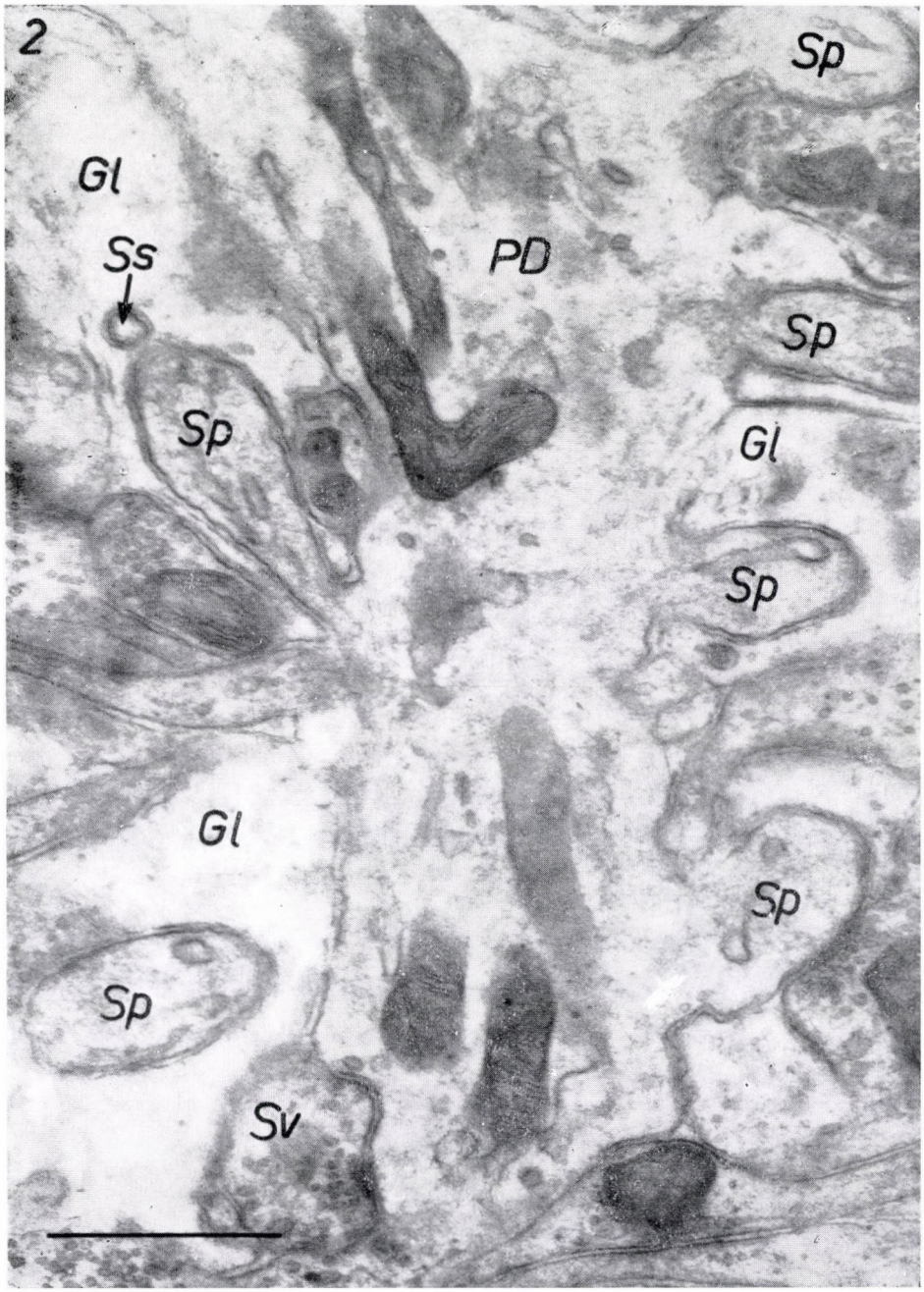


Fig. 2. Same as Fig. 1, note complete glial *(Gl)* envelope of the dendrite and nonsynaptic surface of the spines *(Sp)*. Small secondary spine *(Ss)* is seen to bulge out from left upper spine. At lower left relatively rare direct synaptic attachment to dendrite surface of axonal profile, containing synaptic vesicles *(Sv)* is seen

Fig. 3. Transverse section of small Purkinje neuron dendrite *(PD)*, with radial arrangement of spines *(Sp)* most having synaptic attachment to surface of axonal profiles. Spine right above embedded into invagination of axon. Ringed arrow at lower left points to invaginated spine profile, which is probably secondary protrusion of neighbouring spine. Note endoplasmic sacs in upper left spine, resembling somewhat the "spine apparatus" in the neocortex, and "vesicles" in spine at lower right, which might be mistaken for synaptic vesicles, but more probably are transversally cut endoplasmic tubuli are seen in the spine on Fig. 5

Fig. 4. Oblique section of small Purkinje neuron dendrite *(PD)*. From upper spine *(Sp)*, having one synaptic attachment to parallel fiber at its right side, smaller secondary spine *(Ss)* is seen to bulge through glial space *(Gl)* and it is probably the same secondary spine profile that is seen to attach itself to axonal profile at above left

form widening of the parallel fiber. Wherever the plane of the section is fortunate enough (Figs. 5 and 7), it becomes quite clear that the presynaptic element is in fact only a fusiform widening of the granule cell axon, containing synaptic vesicles from where the fiber itself continues in both directions. The picture in most cases looks very deceptive so that one often gains the impression as if the presynaptic terminals were short side branches of the parallel fibers. We do not want to deny completely the possibility of such relations, but do not think it to be the typical type of synapse. Some of the preterminal axons that have an entirely different course from that of the parallel fibers might correspond either to outer star cell axons or climbing fibers. With the climbing fibers we shall deal in another paper separately, the terminal parts of the stellate cell axons could safely be recognized only on the basis of plastic reconstruction and probably using secondary degeneration in appropriately designed experiments. From Golgi pictures, however, we have got already earlier the impression [13] that the outer stellate cell axons might probably establish the relatively few direct synapses on the smaller Purkinje dendrites (Fig. 2). The Purkinje dendrite spine, in most cases, is only slightly impressed into the parallel fiber (Figs. 1, 3 and 4) but may be embedded deeply into an invagination of the granule axon surface membrane (Figs. 3 and 5) The details of the synaptic contact correspond exactly to the descripton by GRAY [5] of the 1st type of the synaptic contacts. As already mentioned by GRAY [6] there is no spine apparatus in the cerebellum in the sense as in the cerebral cortex [4].

(ii) *Secondary branches of the Purkinje dendrite spines* can be observed very regularly (Figs. 2, 4, 6, 9 and 10). They are branching off from the ends or sides of the main spines as small spheroid or elongated bulges of extremely light structure and are seen always surrounded by the watery plasma of glial elements. Traversing through the glial profiles they establish regular type 1 synaptic contacts with parallel fibers, mostly by simple attachment (Fig. 10) or occasionally by protruding into invaginations (Fig. 3) of the axons. The division of dendritic spines or gemmulae into secondary branches can be seen occasionally also by the light microscope in Golgi preparations of various types of neurons, in the Purkinje neuron spines it appears, however, that secondary branchings of the short rather stout spines is a regular structural arrangement [see 14]. From the functional viewpoint it is important to mention that the secondary spine branches, as a rule, establish synapses with another axon than the primary spine is having contact with. It is noteworthy that the number of secondary spines appears to be extremely variable, in one material they may be extremely rare and very abundant in others. It might therefore be rewarding to investigate whether or not this would be connected with previous functions.

(iii) *The relation of the tertiary Purkinje dendrites and their spines to glial elements* is of particular interest. It has already been mentioned that the main

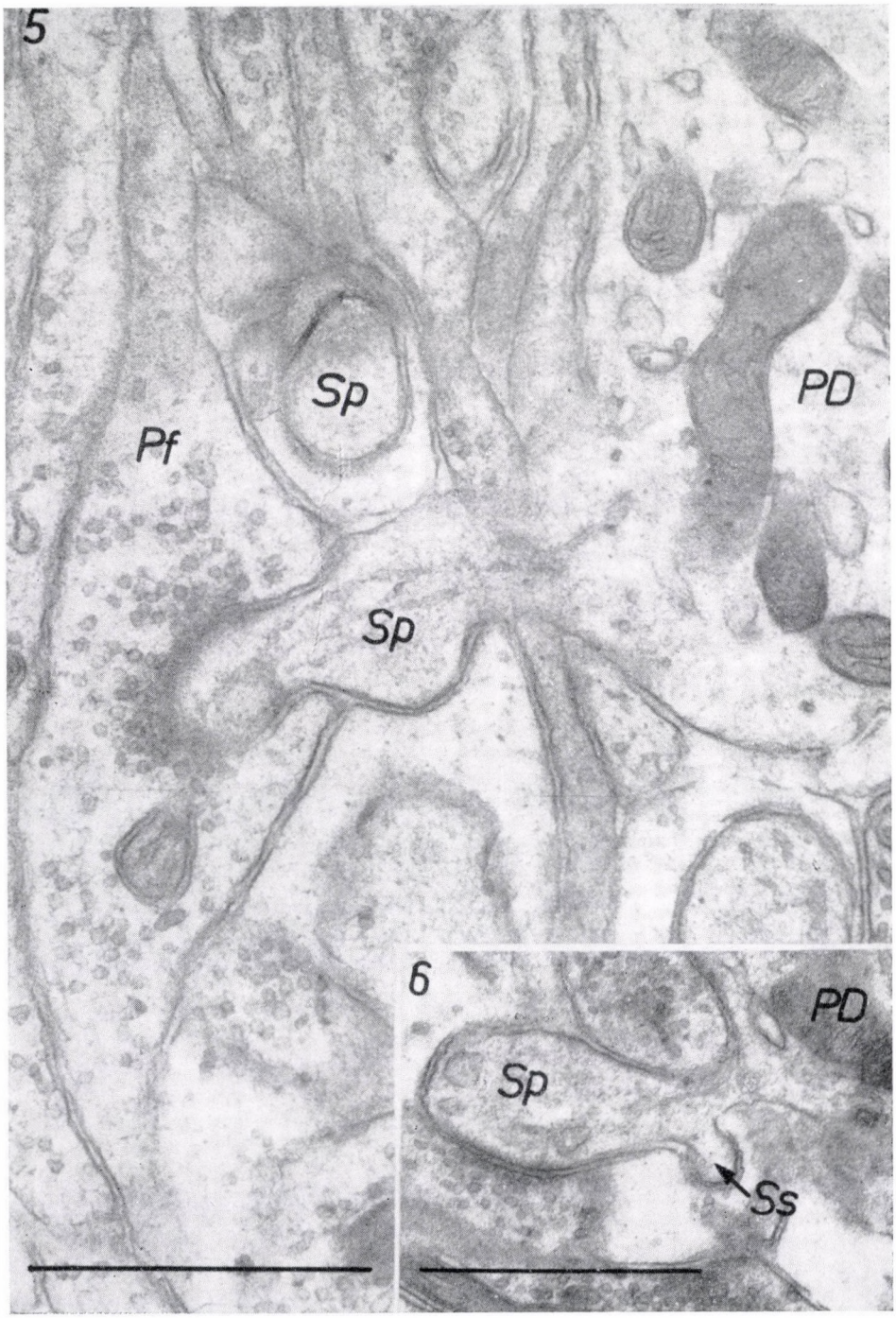


Fig. 5. Spine *(Sp)* of Purkinje dendrite *(PD)* invaginated in fusiform enlargement of parallel fiber [*(Pf)* = granule neuron axon], which is seen clearly to run forward in both directions. Another parallel fiber is seen to cross the root of the spine, somewhat out of section plane, and to have synaptic contact (largely out of plane) with another spine at the upper margin of picture

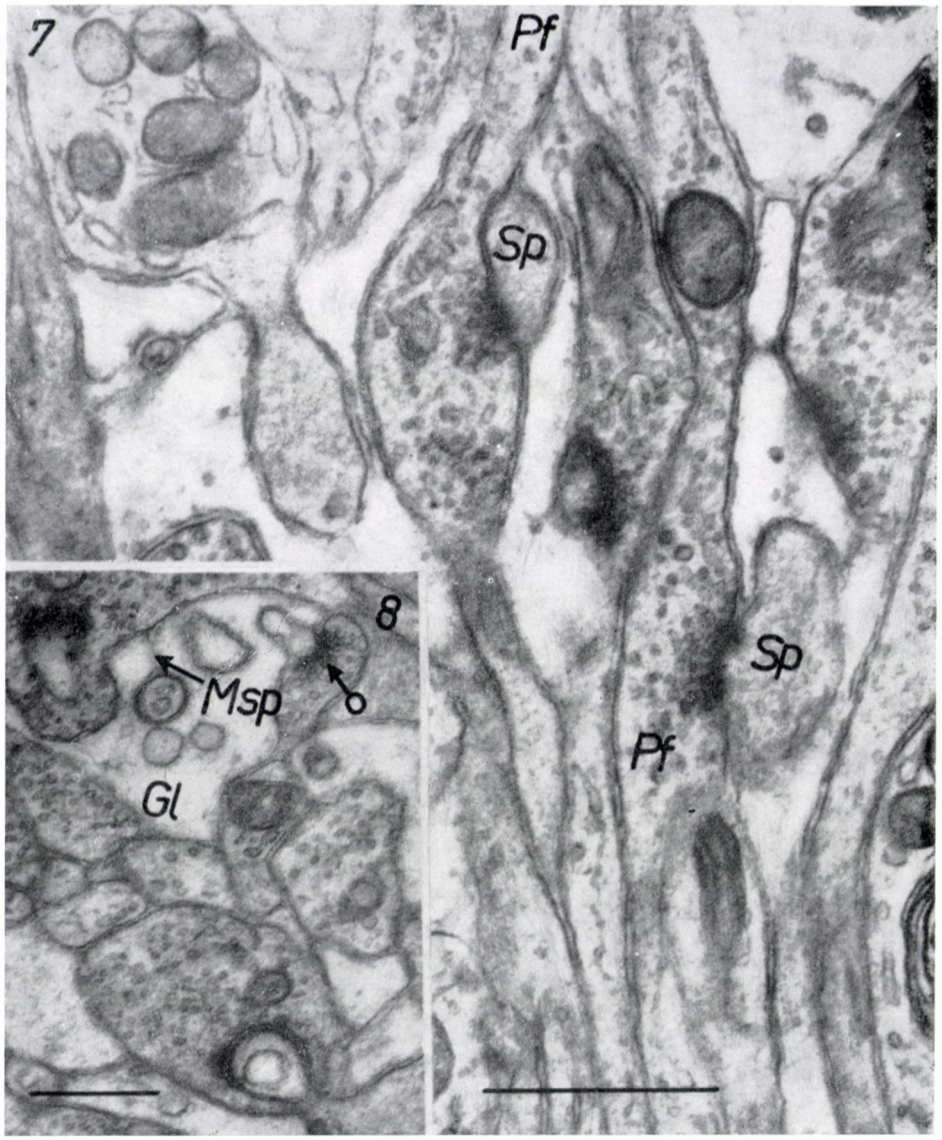
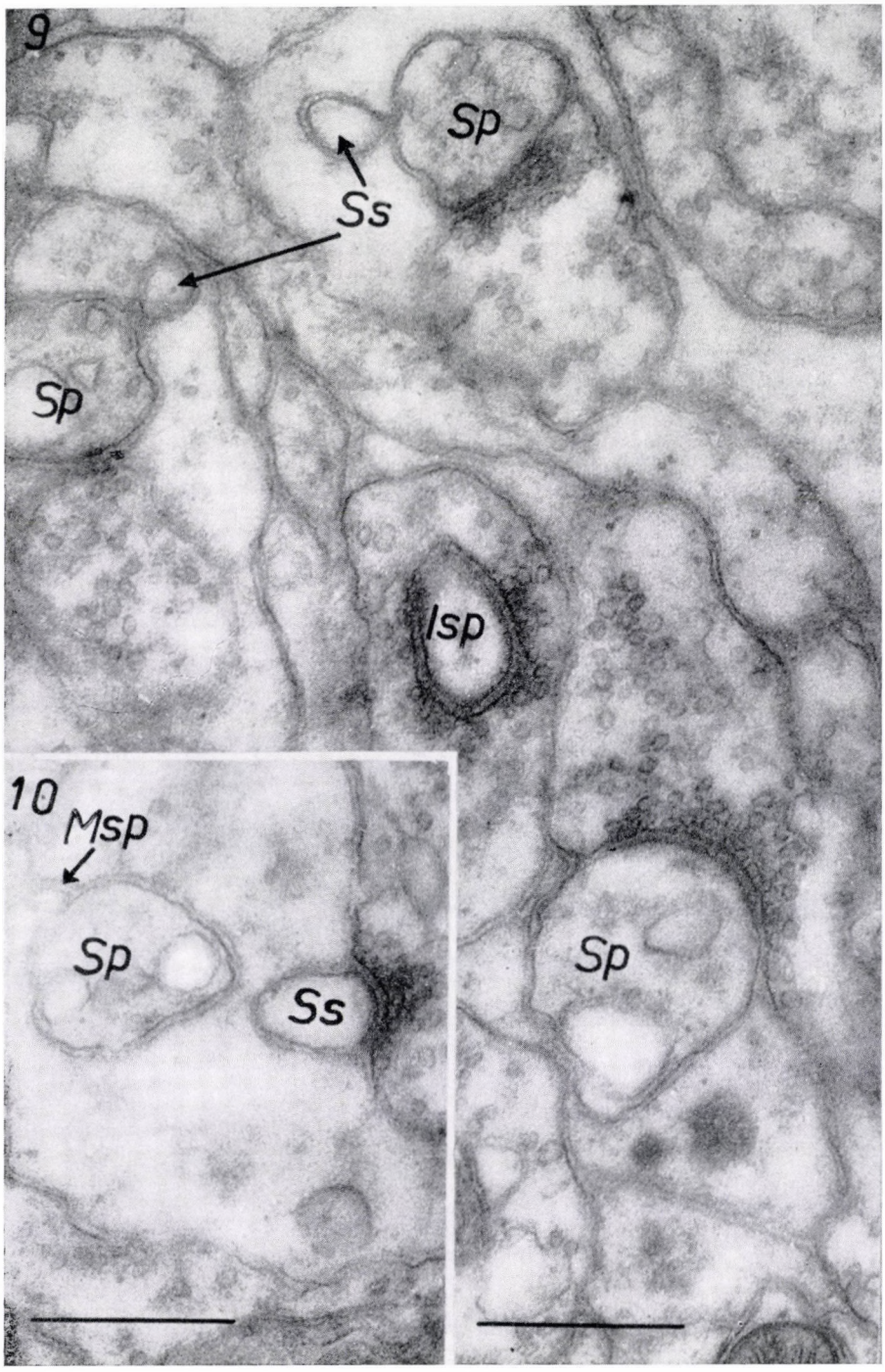


Fig. 7. Parallel fibers *(Pf)*, cut longitudinally, have a fusiform enlargement with accumulation of synaptic vesicles at their sites of synaptic contact with Purkinje dendrite spine *(Sp)*. *Fig. 8.* Watery glial profile enveloping characteristic spine with mesospine *(Msp)* double membrane. Ringed arrow points to synaptic attachment of characteristic light secondary spine to axonal profile. At bottom an invaginated spine synapse containing characteristic terminal vacuole of spine

Fig. 6. Spine *(Sp)* of Purkinje dendrite *(PD)* has a small secondary protrusion *(Ss)* bulging into light glial profile, which due to tangential cutting of the membrane cannot be clearly separated from the axonal profile to its left. This secondary spine will obviously have synaptic contact with axon at lower border, the synaptic vesicles of which begin to appear somewhat out of focus below the letters *Ss*


9
Sp
Ss
Sp
Isp
10
Msp
Sp
Ss
Sp

and also the secondary dendrites of Purkinje neurons, as well as the upper part of the Purkinje cell bodies [12] are almost entirely surrounded by a glial envelope. Due to the many spines and their synaptic contacts this glial envelope is much less regular around the tertiary dendrites, but it can still be recognized as irregular watery cellular profiles surrounding most part of the surface of the dendrites and of the initial part of the spines (Figs. 1, 2, 3 and 4). The uninterrupted double layers bordering these surfaces show clearly that the often seemingly empty outer spaces are in fact intracellular, as indicated also by the occasional mitochondria and endoplasmic structures that appear mostly in widened parts of these profiles. There is no extracellular space whatever in the molecular layer with the exception of the 200 Å wide clefts between the closely packed nerve and glial elements. Smaller spines are often completely surrounded by the glial elements and under these circumstances they may be connected with the extraglial space by double layers of the glial surface membrane, which in analogy to the *mesaxons* of unmyelinated nerve fibers of the periphery might be called *meso-spines* (Figs. 8 and 10). Occasionally also axons are completely embedded into larger glial processes and may have mesaxons, it even may occur that inside such an axon the profile of an invaginated spine is seen. The light secondary spines, as already mentioned, always bulge into the glial spaces, but they do not have, in general, mesospines. Their relation to the glial processes is thus best explained by assuming that they secondarily penetrate through the glial plasma until they reach the surface of some axonal element.

The basket cell dendrites

The basket cells can well be recognized on the basis of their localization in the deeper strata of the molecular layer. They are smaller cells with unusually irregular rugged surface and nuclei with deep invaginations of their membrane that gives them an almost lobular structure. The cell body is peppered with numerous axo-somatic synaptic knobs — mostly of GRAY's type 2 — deeply embedded into the depressions of the cell surface. From considerable differences in their structure these axo-somatic boutons might be suspected as belonging to different kinds of neurons. Some of them may be contacts "de passage" of parallel axons, but this is difficult to decide because of the extreme irregularity of the cell surface. This question, therefore, would need

Fig. 9. Higher magnification of Purkinje spine synapses. Most spines *(Sp)* contain terminal vacuoles, which continue into and fill up almost completely the secondary spines *(Ss)* bulging out from the spines. In the center invaginated spine synapse *(ISP)*. Scale 0.5 micron

Fig. 10. Same as Fig. 9, with pale mesospine *(Msp)* double membrane. Almost hollow secondary spine *(Ss)* has typical Gray's 1 type synaptic contact with axonal profile. Spine and secondary spine are completely enveloped by watery glial process. Scale 0.5 micron

a more thorough investigation by light and electron microscope studies combined with experimental degeneration. We can perhaps revert to it in a forthcoming paper dealing with the synapses of climbing fiber and recurrent Purkinje axon collaterals.

The dendrites of the basket cells could be traced directly from the cell bodies so that they can be identified with fair security. They are of considerably higher density than Purkinje dendrites of equal size, contain numerous dark strongly elongated mitochondria often very densely packed along the axis of the dendrite. Another characteristic feature are flat endoplasmic sacs predominantly localized in the periphery of the dendrites (Figs. 11 and 13). Although the larger Purkinje dendrites have similar structures, the spheroid sacs of their spiny branchlet very clearly differ from the flat endoplasmic sacs of the basket dendrites. The spines of these dendrites are most characteristic, being unusually long, rather slim and of much denser structure than the Purkinje neuron spines (Figs. 11, 12 and 13). They are spaced also at much larger and less regular distances, and because of their curved courses are only rarely seen in the same section plane along their whole length. The basket dendrite spine establish synapses also mainly with parallel fibers, in which they are often seen to be invaginated (Figs. 11 and 13). The numerous invaginated synapses seen everywhere in the neighbourhood of the basket dendrites (Fig. 11) also indicate that large part of their spines have this type of synapse, which is rather rare in the case of the Purkinje spines.

Outer stellate cell dendrites

The stellate cells can be well recognized, being the only nerve cell bodies situated in the outer part of the molecular layer. The perikarya are spheric with very smooth outlines and a regular spheric nucleus having but one or two deeper indentions of its surface. The parallel fibers curve around the cell bodies in immediate apposition to the cell surface, having, however, no synaptic contacts at all with the cell body. There can be found occasionally few axo-somatic boutons on the stellate cell bodies, but these are relatively large, so that it seems improbable that they should belong to parallel fibers. Initial parts of dendrites emerging from the cell bodies could be traced for short distances only, probably in consequence of their tortuous course. Being devoid in their initial parts of spines and lacking also other characteristic features, we were unable to distinguish their profiles from those of other dendrites, which in the outer part of the molecular layer gradually lose their characteristic

Fig. 12. Same as Fig. 11 to show dense packing of mitochondria in basket dendrite *(BD)* and dense longitudinally cut spine *(Sp)* containing flattened endoplasmic sacs, that might resemble the spine apparatus of neocortical spines

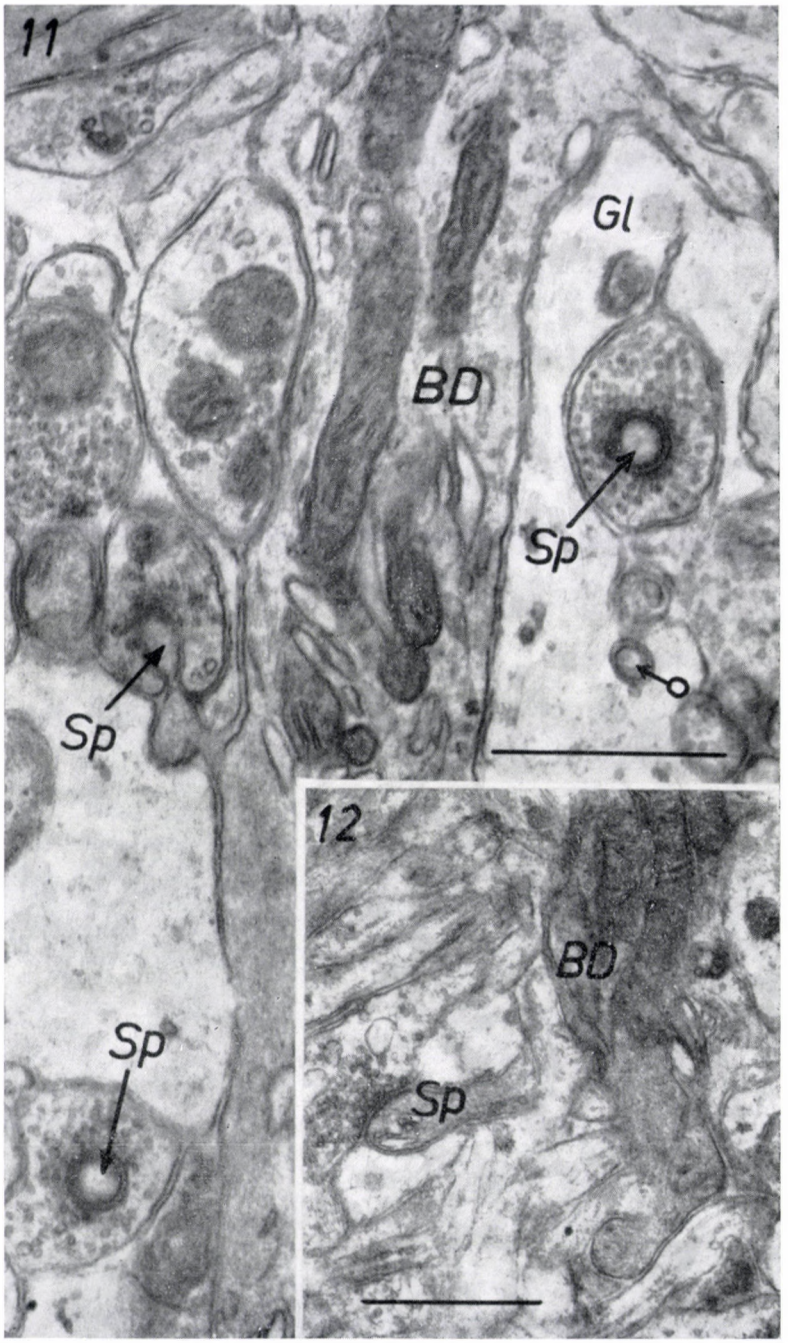


Fig. 11. Dense basket neuron dendrite *(BD)* with few irregularly spaced long thin spines *(Sp)* which are almost invariably deeply invaginated into parallel fiber profiles. Ringed arrow points to transversally cut spine piercing through the glial envelope *(Gl)* of the dendrite

13
Sp
BD
Sp

features. In the superficial part of the cortex it practically never occurs that any dendrite is cut longitudinally in sufficient length to give any information about the spacing and arrangement of the spines. Thus one of the most important criteria of the recognition of the dendrites is absent. But as the dendrites of the stellate cells have, according to Golgi pictures numerous delicate long spines, quite similar to those of the basket cell dendrites, and as the vast majority of synaptic contacts in the outer part of the molecular layer are those of smaller dendrites or dendritic spines established with parallel axons, there is no doubt that the great majority of synapses on the dendrites of star cells are established with the granule cell axons.

Golgi cell dendrites

As known from the classical descriptions, the Golgi II type cells of the cerebellar cortex, fairly regularly localized in a row immediately beneath the Purkinje cell bodies, have two kinds of dendrites. One group descends into the granular layer, where their branches are involved in the cerebellar islets, having thus synaptic contacts with the mossy afferents. The other group of dendrites — more numerous in general than the former ones — run between the Purkinje cell bodies into the molecular layer where they ascend almost to the pial surface in a characteristic stiff arborization pattern. The smaller dendrites have few also rather stiff and relatively long spines. — Since these Golgi neuron dendrites are the only ones that traverse the ganglionic layer, we looked very thoroughly for dendrites in the regions between neighbouring Purkinje cell bodies. There was found one kind of dendrite indeed that ascend through the ganglionic layer and by this criterion could very well belong to the ascending dendrites of Golgi II type cells. These dendrites are of unusually light plasma structure, have few mitochondria of extremely varying size and small spheroid endoplasmic sacs often connected by longitudinal filaments or thin tubuli so that they often appear as rows of beads of irregular size. Almost the entire surface of these dendrites, both in the ganglionic layer and immediately beneath is covered by synaptic terminals in an uninterrupted row. Most of these synaptic terminals have very few synaptic vesicles accumulated at a small region of the attachment of the endings to the dendrites and a thickening of the postsynaptic membrane is found regularly opposite these accumulations. Unfortunately these dendrites are lost from sight in the molecular layer, so

Fig. 13. Transversally cut basket neuron dendrite *(BD)*. Note characteristic dense structure and close packing of mitochondria, as well as flattened endoplasmic sacs near the surface. One of the long thin spines *(Sp)* is longitudinally cut in its entire length up to its end where it is invaginated into an axonal profile. Another spine *(Sp)* left above has synaptic attachment to fusiform enlargement of parallel fiber. Transversally cut round profiles in upper part of the photogram are ascending granule neuron axons before their "T"-shaped branching.

that nothing can be said at present about their synaptic contacts, although it is highly probable that they have synapses also mainly with parallel fibers. From Golgi pictures at least this conclusion emerges almost with certainty and CAJAL [2] had no doubt that the main presynaptic system of Golgi neurons in the cerebellar cortex are the granule axons. This question has recently been discussed by one of us [14] and being largely beyond the scope of this paper, due to the several kinds of axo-somatic synapses on the Golgi cells and the strange synaptic endings in the entire length of the proximal parts of the ascending dendrites, the whole problem of the Golgi cells is better separated from this material in order to be discussed in detail on another occasion.

Discussion

Electron microscopic observation thus fully supports the classical description of the molecular layer as a synaptic system in which the axons have repeated synaptic contacts — "de passage" — with dendritic elements that they happen to cross at close distance. The spines appear to be simply a device to effect this crossing over contact and to increase the distance at which a certain dendrite can get into contact with axons passing by. It would certainly be interesting to make exact calculations on the factor by which the spines can increase the number of parallel fibers the average dendrite can establish contact with per unit length. In the case of the Purkinje dendrites this is at least 2 if the secondary spines are left unconsidered, with secondary spines another factor of at least again 2 has to be introduced so that the total factor would be 4. It were, however, an oversimplification if one would consider the spines only as a device to increase opportunities of contact. A considerable part of dendritic surface not only of the larger, but also of the tertiary dendrites is left unused for synaptic purposes simply by being blocked through glial tissue. Even if a parallel axon happens to be in immediate apposition to the dendrite surface (Fig. 1), no specialized synaptic contact is established between the two, however, on the outside of the same parallel fiber a reclining spine of the same dendrite has a clear synaptic contact. Similarly the parallel fibers, curving around the smooth bodies of the outer stellate cells, do not have synaptic contacts with them, but the same axon on its opposite side has numerous real contacts with dendritic spines. This suggests two things: (i) that nerve elements must be extremely selective with respect to what kinds and what parts of neurons they establish or avoid to have synaptic contact with and (ii) there must be some important cause or consequence, functional or other, of the fact that certain elements have synapses with spines only, others with the dendrite surface, and again others with cell bodies. There are rather rare occasions that an axonal profile establishes a direct synapse with a Purkinje dendrite (Fig. 2), but in neither of these cases could we convince ourselves that this

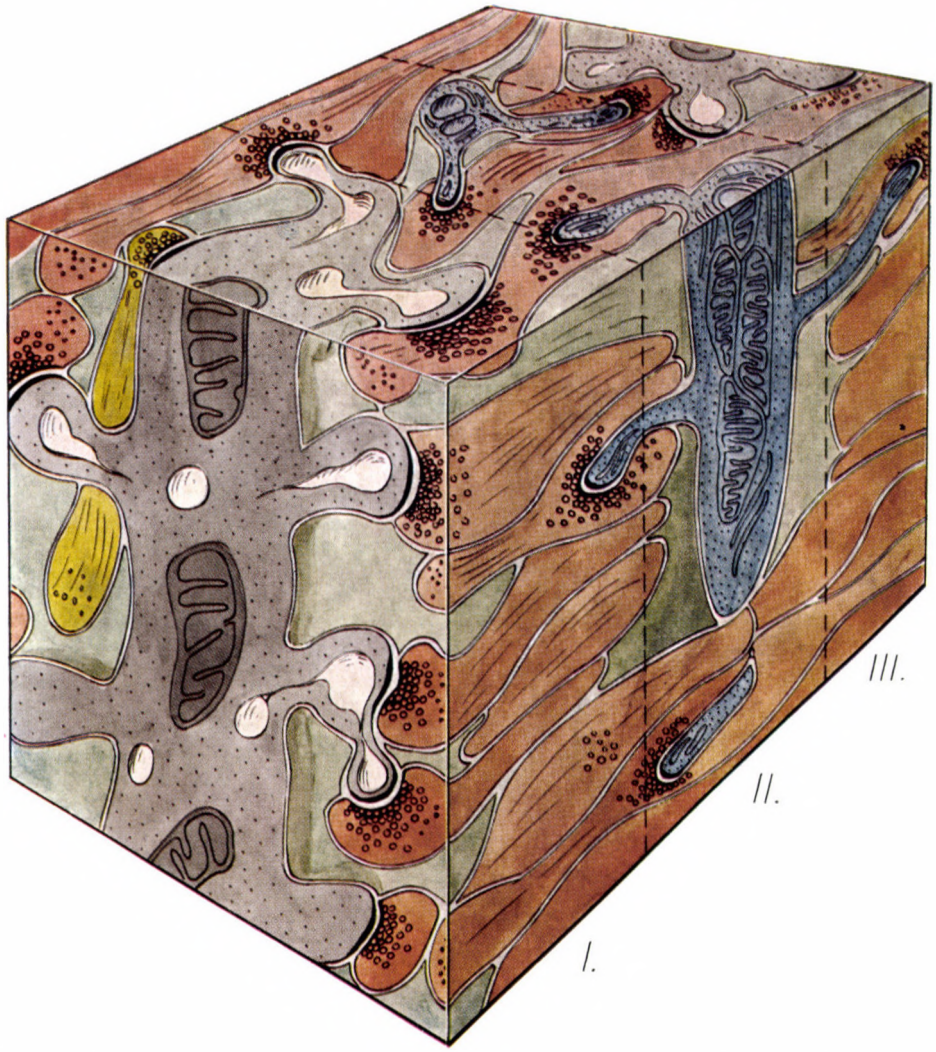


Fig. 14. Stereoscopic diagram of the cerebellar cortex molecular layer to show synaptic relations between parallel fibers (red), Purkinje neuron dendrites (grey), basket neuron dendrites (blue) and outer stellate neuron-axon (yellow). Glial processes surrounding the dendrites are shown in green. With the exception of the outer stellate axon synapses all other synaptic contacts are "crossing-over" synapses, with dendritic spines or secondary spines. The slabs separated by dashed lines indicate territories for the branching of Purkinje dendrites (I and III) and for basket dendrites (II), which occupy alternating transverse discs of the cerebellar folium

was a parallel fiber. It always looks as belonging to a fiber of vertical or irregular course. Sometimes these axonal profiles are rich in neurofilaments, which could indicate that they belong to climbing fibers.* Others are probably outer stellate cell axon endings, which we have already on the basis of light microscope studies on Golgi material thought to be most likely in direct contact with the dendritic surface [13]. The same considerations are valid for all other neurons having dendrites embedded into the molecular layer. According to Golgi preparations all of them have characteristic spines, although not in the same abundance than the Purkinje dendrites. The basket neuron dendrites can clearly be separated from the Purkinje dendrites by their denser structure and their thin elongated spines. According to light microscope observations on Golgi preparations the same must hold true for stellate cell dendrites as they have very similar long and thin spines [14] and also for upper (i.e those that enter the molecular substance) dendrites of the Golgi neurons. Golgi preparations with simultaneously stained Golgi neurons and parallel fibers very impressively suggest that they have the same crossing over synaptic contacts that have the parallel fibers with all other dendrites arborizing in the molecular layer. The important functional consequences of this principal agreement in synaptic contacts of all neurons having dendritic arborizations in the molecular layer have been considered in detail [14] and have led to interesting conclusions, which could be tested upon their validity by suitable electrophysiological experiments. These synaptic relations are diagrammatically shown in Fig. 14, which tries to convey a plastic impression of the finer architecture of the molecular layer.

These considerations of the axon-spine synapses of the cerebellar cortex are essentially valid also for other regions of the central nervous system. The ultrastructure of the axon-spine synapse has been very clearly described first by GRAY [4, 6] and with minor differences these descriptions hold true in almost every part of the CNS. Axon-spine synapses very often belong to the "*invaginated type*" of synaptic contact [6] always with the invagination of the dendritic part into the axonal element. — In what we cannot agree entirely with the earlier descriptions is that the presynaptic element is considered generally — whether this is mentioned explicitly or not — as some terminal knob of the axon. From a careful survey of a very large number of preparations with appropriate plane of sectioning it becomes clear that this is not so in the cerebellar cortex. Here — although it might look like that in many instances, in consequence of the relatively small probability that the fusiform thickening of the parallel fiber, which has the contact with the spine, and its thinner

* We have some reason to believe that climbing fibers are particularly rich in neurofilaments, which might be used under certain circumstances as a criterion for their recognition. Unfortunately there is another element even more neurofilamentous: the basket neuron axon. These questions will be dealt with in forthcoming papers.

continuations on both sides are in the same plane — there can be no doubt that in the overwhelming majority of axon spine contacts this is no terminal contact from the viewpoint of the axon but a contact "de passage". Electron microscopic investigation thus fully supports the notion of the "crossing over" synapse which was so forcibly suggested by the Golgi picture, so that CAJAL never appeared to have any doubt in its reality. The arrangement of nerve elements is nowhere as regular as in the molecular layer, so that it cannot be expected that, without making some cumbersome plastic reconstructions, one would clearly recognize such relations in other parts of the CNS. However, in analogy to the molecular layer of the cerebellum it is most probable from the Golgi picture of many other regions that axon-spine synapses are in general the device by which crossing over contacts are established. In the cerebral cortex, especially of course in the zonal layer, the situation between the tangential axonal plexus and the branches of the apical dendrites are principally the same as between dendrites and parallel fibers in the cerebellar cortex. An entirely similar situation is prevalent in many parts of the hypothalamus, especially in the ventromedial nucleus where the dendrites have a rich system of very delicate long spines and where an extremely delicate terminal plexus of axonal branches exists. From relative rarity of true axonal endings in the Golgi pictures and the relations between the elements of the axonal plexus to the dendrites and dendritic spines [16] it has already been inferred from light microscopical studies that the majority of axon-spine synapses in the hypothalamus might be of this "crossing over" contact type. From recent investigations into the arrangement of neurons in the substantia gelatinosa [15] it appeared that its structure highly resembles that of the molecular layer of the cerebellum. It has in addition to the mostly radially orientated (i.e. perpendicular to the dorsal border of the S G.) terminal arborizations of extrinsic (primary afferent and other) systems a longitudinally orientated intrinsic system of very delicate unmyelinated axons, which according to Golgi preparations appeared to be in exactly the same relation of "crossing over" synaptic contacts with the dendrites arborizing in this substance, as the parallel fibers are with the molecular layer dendrites. Most recent yet unpublished observations by the electron microscope fully support these views and are showing that contact in this crossing over synaptic system is also mainly established by dendritic spines embedded into invaginations of the axons.

We are arriving thus at the conclusion that the axon-spine synapses can be considered as the main device for effecting synaptic contact between two nerve elements (axon and dendrite) that cross each other at relatively small distance. It is most impressive to think over this from the viewpoint of the economy in the architecture of central grey matter. Apart from the fact, that the number of synapses per unit length of the dendrite is increased through the spines by a factor of 2—4 the synapse by overcrossing assures an immense

degree of simplicity in comparison with the complexity of arrangement if any synaptic contact would require an individual side branch, terminating in a separate end-knob of the presynaptic axon. Simply by running a few millimeters in straight direction, the preterminal axon branch can overcross the homologous dendrites of a whole group of neurons having a synaptic contact with all of them or at any rate with a significant fraction of the group, without the necessity to give off a single side branch. This pattern of contact thus may secure a high degree of "divergence" with the possible minimum of axonal branching. On the other hand this arrangement of neurons can assure also a very high degree of convergence, if the axons involved are individual processes of neurons and not branches of a brush-like arborization of axons into numerous terminal fibers of parallel course. Although this latter case may occur occasionally, most of the terminal axons involved in crossing over synapses are in fact either single fibers resulting from a "T"-shaped arborization — as it is the case with the parallel fibers in the cerebellum — or the branches of generally not too rich arborizations. As it emerges from the calculations of Fox and Barnard [3], the number of spines per Purkinje neuron may be around 60.000, which with the secondary spines might bring up the number of possible contacts to 120.000 parallel fibers from the roughly 250.000 calculated by Fox and Barnard to cross the arborization of an individual Purkinje cell. This would mean additionally that the parallel fiber may have a synaptic contact with every second of the Purkinje cells, the dendritic territory of which it crosses. We will not go into the functional conclusions that suggest themselves from these calculations, as they have been considered already in detail in other papers [12, 14]. On the basis of these speculations the high regularity fo the specific orientation in space, mainly of dendrites but to some extent also of terminal axons becomes very meaningful and it is by no means surprising if this regularity — as well as the occurrence of axon-spine synapses — is most characteristic for integrative centers (cerebral cortex, cerebellar cortex, tectum, hypothalamus) in which information is processed by mechanisms involving very high degrees of divergence and convergence in combination. It is of course in highly different form that this principle of neuron coupling is being used in the different centers. In the cerebellum the higher units of integration are represented by strips of simultaneously excited matrices of Purkinje cells of about 3—4 mm length — running parallel with the long axis of the folium — and having two side fringes of inhibited Purkinje cells [12, 14]. These units, however, are not fixed anatomically, but are a pattern of the main afferent inflow through the mossy fibers. In the cerebral cortex, especially in primary sensory regions, the higher integrative units are columns of cells running radially through the entire depth of the cortex [7, 8], probably determined by common input through specific afferents, but especially in the superficial regions united by the zonal (1st layer) axonal plexus, which

is mainly fed by Martinetti type neurons of the 5th and 6th layer of the same column. The synaptic mechanism by which the apical dendrites of a great many of stellate pyramid or pyramid neurons are linked functionally are also mainly of the crossing-over type synapse [11]. This mode of synaptic arrangement is not restricted to higher integrative centers, as it occurs also in the spinal cord. The substantia gelatinosa is, as mentioned, essentially a synaptic system with crossing over synapses between a longitudinally orientated axonal plexus and predominantly radially orientated dendrites [15]. But this system evidently is not a simple device of forwarding information with perhaps some minor transformation. It is on the contrary a neuron system able to build up gradually, by an extreme wealth of reverberating circuits, long lasting states of excitation, which in turn can modify the direct transmission of sensory impulses through the dorsal horn by acting upon the dendrites of large posterior horn neurons embedded into the substantia gelatinosa and having crossing-over synapses with its longitudinal axonal system.

A quite unexpected feature in the architecture of the molecular layer of the cerebellar cortex is the very close relation between the immediate pre- and postsysnaptic nerve elements and the glial tissue. It is true that there is very little extracellular space in almost all kinds of CNS grey matter, with the exception of the cleft system, about 200 Å wide, separating the closely adjacent cellular membranes of the several tissue elements. The glial component, however, of the central nervous tissue is rarely predominant, generally because of the very close packing of the nerve elements, only around blood vessels and myelin sheaths are larger profiles of glial processes seen in significant number, as well as of course in the perinuclear regions of the glial cells themselves. The molecular layer of the cerebellar cortex seems to be quite exceptional with respect to the relative large fraction of cross sectional area occupied by watery profiles that look almost empty and could easily be mistaken for extracellular spaces if it were not for the clear cellular membranes that surround them everywhere. It is rather exceptional that one finds mitochondria and other plasmatic differentiations, and generally only in the larger ones of these watery profiles, which clearly identify them as glial processes. The glial profiles are accumulated around the dendrites of both, Purkinje neurons and others, they do not, however, penetrate between the groups of parallel fibers, which, if not in the immediate neighbourhood of a dendrite, are packed closely together without any interception of foreign cellular material. The dendrites, on the contrary, are almost in their entire surface covered by the watery glial profiles, which practically block them from direct contact with other nerve elements. Only once a while is a nerve terminal admitted to have immediate synaptic contact with the dendrite surface proper. As mentioned, these synapses are probably those of climbing fibers in the case of the Purkinje dendrites and of outer stellate axons in Purkinje as well as basket neuron den-

drites. The spines of the dendrites pierce through the glial envelope until they reach the parallel fibers outside. It is, therefore, a very common picture to see the transverse or oblique section of a spine and even more often of secondary spines completely surrounded by the watery glial profile and a clear double cell membrane, one of them being the membrane of the spine and that of the glial cell the other. But it also occurs quite often that a part of a parallel fiber or dendrite is so completely surrounded by a glial profile that it is connected with the outer intercellular space only by a double glial cellular membrane, mesaxon or mesodendrite. In some cases even the spines can have similar relations to the glial cells so that clear "mesospines" can be seen in our preparations. With respect, however, to the relative rarity of mesospines under circumstances, when one should expect to find many of them, rather suggests that the outgrowth of spines and especially of secondary spines must be a secondary process, occurring only after the fundamental spatial arrangements between dendrites, axons and glial elements — it is apparently mainly the Fananas glia that is involved here—have been settled, so that the spines have literally to pierce through the glial envelope of the dendrite in order to get into contact with the outer axons. Whether or not this process of establishing additional synapses by secondary spines could be a matter of dynamic change for example in consequence of increased or decreased functions, should be investigated under appropriate conditions.

This rather extraordinary relation to the glial elements could perhaps explain the difficulties encountered in the application of degeneration methods to the molecular layer. It has been noticed in this laboratory already many years ago [7, 14, 17] that secondary degeneration of axons does not show up in the molecular layer. This difficulty has for long time impeded the recognition of the origin of climbing fibers [17]. It may well be that the large amount of glial tissue and its close relation to the nerve elements may have something to do with the difficulties of histological demonstration of axonal degeneration fragments. There is indeed some inverse relation between the amount of glial elements as well as the density of packing between glial and nervous elements and the ease or effectiveness with which degenerated axonal fragments are stained by Bielschowsky modifications or by the Nauta-Gygax procedure. The staining of degenerated axonal fragments is most easy in loosely arranged types of grey matter of those areas that have been called as regions of reticulated structure, whereas staining is always incomplete or difficult in the dense gelatinous types of grey matter (molecular layer, outer parts of cerebral cortex, substantia gelatinosa, hypothalamus). It would be interesting to compare these regions with respect to glial architecture, density of packing of elements, intercellular spaces etc., to the more reticulated and loosely packed types of grey matter, where as e.g. in the anterior horn of the spinal cord there seem to occur true intercellular spaces of quite considerable width.

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DIE »ÜBERKREUZUNGSSYNAPSE«: EINE ELEKTRONMIKROSKOPISCHE STUDIE DER MOLEKULARSUBSTANZ DER KLEINHIRNRINDE

Die Feinstruktur der Synapsen, insbesondere jener zwischen Dendritendornen und Parallelfasern, wird vorwiegend in Hinblick auf die allgemeinen Prinzipien synaptischer Verschaltung studiert. Die die überwiegende Mehrzahl der Synapsen der Molekularschicht liefernden Dornensynapsen werden als charakteristisches Beispiel des synaptischen Kontakts durch »Überkreuzung« gewertet, eine Anordnung, die geeignet ist, ein Höchstmass an Konvergenz und Divergenz der Schaltung mit möglichst geringster Aufzweigung der zuführenden Axonen und einer damit verbundenen möglichst einfachen Architektur der grauen Substanz zu verbinden. Es wird versucht, die kleineren Dendriten von Purkinjezellen, Korbzellen und äusseren Sternzellen im elektronenmikroskopischen Bild zu identifizieren, was unerlässlich für ein besseres Verständnis der neuronalen Schaltung und Aufarbeitung von Erregungsprozessen in der Kleinhirnrinde ist. Besondere Beachtung wird den ungewöhnlich engen Beziehungen zwischen gliösen und nervösen Elementen der Molekularschichte gewidmet, und deren mutmassliche Bedeutung, sowie allgemein auch jene von Unterschieden im feineren Bau des zentral nervösen Graus erörtert.

ПЕРЕКРЕЩЕННЫЙ СИНАПС: ОЧЕРК ПО ЭЛЕКТРОННОМИКРОСКОПНОМУ СТРОЕНИЮ МОЛЕКУЛЯРНОГО СЛОЯ КОРЫ МОЗЖЕЧКА

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Printed in Hungary

Kiadásért felel az Akadémiai Kiadó igazgatója — Műszaki szerkesztő: Farkas Sándor

A kézirat nyomdába érkezett: 1964. VI. 19. — Terjedelem: 10,24 (A/5) iv, 58 ábra (13 színes), 3 melléklet

64.59090 Akadémiai Nyomda, Budapest — Felelős vezető: Bernát György

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Acta Biol. Hung., **15** (2), 119—128 (1964)

THE EFFECT OF CHOLINOTROPIC DRUGS ON THE RHYTHMIC ACTIVITY OF GLOCHIDIA OF FRESH-WATER MUSSEL (ANODONTA CYGNEA L.)

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(Received September 11, 1963)

Synopsis

The effect of various drugs was investigated on the spontaneous rhythmic motoric activity of fresh-water mussel's larvae (*Anodonta cygnea* L.) and on the increased and lasting activities produced by KCl and tryptamine. It was found that the glochidia responded to most substances tested (atropine, hexamethonium, TEAC, nicotine, curare, TMAJ) non-specifically with an initial increase in activity. None of these agents produced tonic contraction. Atropine, TEAC and nicotine blocked the lasting activity produceable by KCl. Hexamethonium was found to be less inhibitory, and curare was ineffectual. The effect of K^+ was potentiated in the presence of TMAJ. The effect of tryptamine was inhibited to a small extent by atropine, TEAC, TMAJ and curare. Hexamethonium and nicotine were ineffectual in this respect. These results suggest that the effects of KCl and tryptamine are realized by way of two different mechanisms, and further that the spontaneous rhythmic activity is of myogenic origin, and is based on the nature of the embryonal adductor.

Introduction

It is known that the larvae (glochidia) of fresh-water mussel (*Anodonta cygnea*) living in the gill of the parent display a rhythmic activity, which is connected with the function of embryonal adductor.

In the course of previous studies [2, 3] it was established that the frequency of rhythmic activity of larval adductor can be greatly and lastingly increased by KCl and tryptamine. A lasting tonic contraction is produced in a considerable number of glochidia within few minutes after the addition of high concentrations of KCl and tryptamine.

The present work deals with the effect of various drugs. In the course of these studies it was attempted to examine the modes and degree of the action of these drugs on the "spontaneous" rhythmic activity of adductor of glochidia as well as the influence of the same substances on the lastingly increased activity produced by KCl and tryptamine. The choice fell on substances affecting cholinergic transmission processes (atropine, hexamethonium, nicotine, TMAJ, TEAC, d-tubocurarine). From the results it was expected to obtain certain information about the effect of these agents on the rhythmic activity of muscular elements of glochidia, all the more because their influence

on nervous and muscular elements of onto- and phylogenetically more developed animals is already known. It was also aimed at to find whether the adductor of glochidia is activated with or without the intermediation of nervous structures. With regard to the fact, that some drugs may have a more positive effect on nervous structures than on muscular elements and conversely, it is expected to find an answer to these assumptions.

Material and method

The glochidia can be obtained from the lamellae of the gill of the parent from October to March. They were released from the mucuous material in which they are imbedded, by washing in tap water. Thereafter they were selectively grouped with a pipette of suitable dimension into 25 individuals, and the number of their rhythmic contraction was counted under binocular microscope. Control investigations were performed on glochidia placed into tap water, and the number of rhythmic contractions was measured five times at intervals of one minute. The agents in question were added to the animals after the tap water was drained and both the frequency of rhythmic action and the number of tonic contractions i.e. closures of glochidia were noted in every single minute during 10—30 minutes. The effect of the agents were studied on four groups of glochidia each (100 individuals).

Considering the fact that K^+- and tryptamine-effects may more or less be quantitatively deviating depending on the differences in age and in population of glochidia, the comparative results presented in this paper were obtained from investigations performed in one day and on glochidia originating from one parent.

The substances used were dissolved in tap water. In case two agents were studied simultaneously they were added in one solution.

The results are illustrated in a system of coordinates. Abscissa: time (control measurements for 5 minutes + 10 minutes of examination). Ordinate: number of contractions per minute.

The agents used were: atropinum sulfuricum, d-tubocurarine chloratum, hexamethonium bromatum, nicotinum tartaricum, tetraethylammonium chloratum (TEAC), tetramethylammonium jodatum (TMAJ).

Results

The effect of atropine

Atropine solution of 100 μg/ml concentration causes an inital increase in activity. Later the number of contractions decrease, but still remains five times greater than the control (Fig. 1). No lasting tonic contraction, i. e. no lasting closure was observed at the above atropine concentration. The high lasting activity taking place as a rule on the application of KCl alone, is not observable when using this substance in 1 mM concentration together with 100 μg/ml atropine. In the presence of atropine the number of rhythmic contractions (in a period of 10 minutes) produced by KCl falls to 3—5%. The activity observable in this case does not differ considerably from that induced by atropine alone. It is inferred on basis of these observations that the activity-increasing effect of KCl is inhibited by atropine.

The degree of activity caused by using 100 μg/ml tryptamine and 100 μg/ml atropine together is 30% less than the activity produced by tryptamine

alone and is considerably higher than the activity obtained when adding atropine alone. These results show that tryptamine at concentrations usually yielding high and lasting activity is less effectual in the presence of atropine, though the effect induced by tryptamine is not in the least inhibited to such an extent by atropine as the effect of KCl.

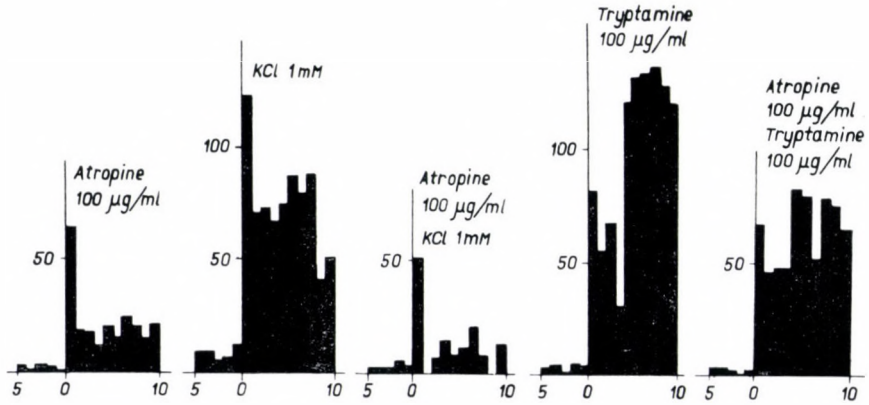

Fig. 1

The effect of curare

It was found that d-tubocurarine in 100 µg/ml concentration does not produce lasting increased activity. In the 1—3 minutes after its application an increased activity — about 30 times, later 10 times greater than the control — is observable decreasing slowly to the level of control (Fig. 2). Lasting tonic contraction of glochidia was not found either.

When adding 100 µg/ml curare to 2 mM KCl solution, the activity of the latter is not essentially inhibited. This involves that KCl is able of producing high activity level also when curare is present at the above concentration (Fig. 2). This agent does not inhibit the lasting tonic contraction caused by KCl either. The same thing holds true when increasing about six times the ratio between the concentrations of curare and KCl (1 mM KCl and 1 mM curare).

Similar phenomena were noticed when using tryptamine (10 and 100 µg/ml) and curare (100 µg/ml) together, i.e. the activity increasing influence of tryptamine does not decrease considerably in the presence of curare and also the tonic contraction of adductor takes place (Fig. 2).

The effect of hexamethonium

Hexamethonium was investigated at concentrations between 20 µg—1000 µg/ml. Threshold is below a 20 µg/ml concentration. The effect of this drug is characterized by a relatively high activity taking place in the first minute

after its addition followed by a decrease in the fifth minute to about the level of control. The same applies to all concentrations investigated. Lasting activity and lasting closure was not observable. The effect of hexamethonium (at concentrations of 100 and 200 μg/ml) on 1 mM (Fig. 3) and 2 mM KCl solutions was also studied. In this case K^+-effect was inhibited and the degree of inhibition depended on the ratio between the concentrations of the two agents. The smaller the ratio between the mol concentrations of KCl and C_6 the smaller is the inhibition.

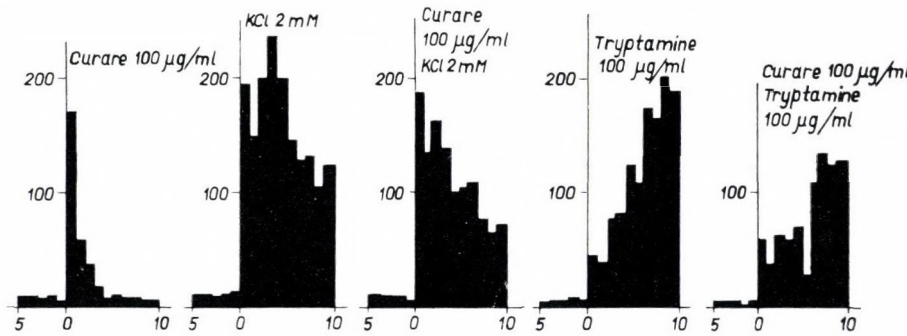

Fig. 2

When using tryptamine solutions of 10 and 100 μg/ml concentrations together with 100 μg/ml and 200 μg/ml hexamethonium each the resulting decrease in activity is insignificant in comparison to the effect produced by tryptamine alone (Fig. 3).

The effect of nicotine

Nicotine was used in 10, 100, 200 and 500 μg/ml concentrations. After a rapid rhythmic activity at start in general for one minute, the activity falls back to the level of control. No lasting closure is produced. Threshold values are obtained below 10 μg/ml concentration.

The effect characteristic of KCl is not observable when adding nicotine to KCl solutions, instead a high activity characteristic of nicotine was recorded in the first minute. Thus nicotine inhibits the lasting activity produced by KCl (Fig. 4).

The stimulatory influence of tryptamine (10 μg/ml and 100 μg/ml concentrations) is not inhibited by nicotine, and a lasting high activity is observable when using these two agents together. When adding nicotine to 100 μg/ml tryptamine solutions the activity occurring from the 7th minute of observation is considerably higher than that induced in the absence of this substance

(Fig. 4). Nevertheless this effect cannot be attributed with absolute certainty to the presence of nicotine, because the same effect was observable also in many cases when using tryptamine alone. The single conclusion that may be drawn is, that nicotine does not inhibit the effect of tryptamine under the conditions investigated.

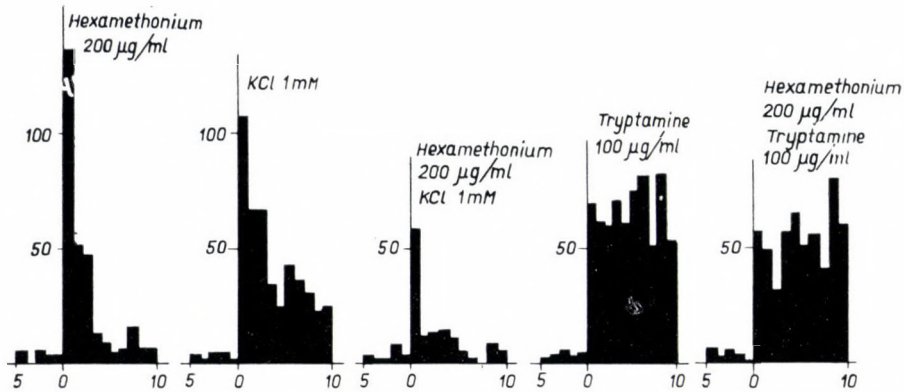

Fig. 3

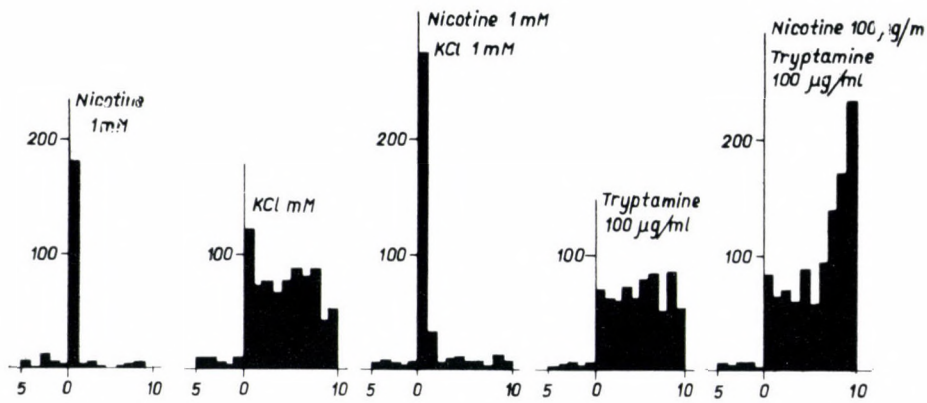

Fig. 4

The effect of TEAC

The activity-increasing effect of tetraethylammonium chloride (TEAC) on the spontaneous rhythmic activity manifested itself in a more positive activity in the first 5—6 minutes of observation, decreasing later to about the level of control. This effect is observable already at concentrations as low as 10^{-4} M. No lasting closure was produced by this agent.

The effect of 1mM KCl solution decreases considerably on the addition of TEAC (1 mM solution). The activity observed in the presence of these two agents is considerably lower than in the presence of KCl alone, but not less than the activity caused by TEAC alone (Fig. 5). Thus in this case the activity-decrease is attributable to the inhibitory effect of TEAC on K^+-effect.

The degree of activity caused by 100 mM tryptamine solution decreases similarly in the presence of 100 μg/ml TEAC. This decrease, however, is considerably smaller than that taking place when influencing KCl-effect (Fig. 5).

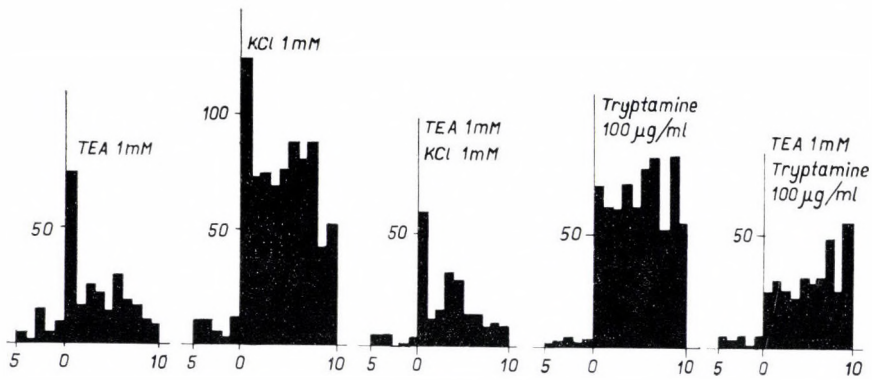

Fig. 5

The effect of TMAJ

When using tetramethylammonium iodide alone at concentrations between 10 μg/ml and 500 μg/ml an initial activity-increase lasting for one or at most two minutes is observable. Thereafter the activity reaches the level of control. Threshold concentration is below 10 μg/ml. The number of contractions registered during 10 minutes increses with the increase of concentration.

TMAJ does not induce lasting closure and the glochidia are open during the time of investigation, closing and opening rhythmically their valves.

When using KCl and TMAJ together (2 mM KCl with 2,5 mM TMAJ, and 1 mM KCl with 1 mM TMAJ) the level of the rhythmic activity induced is very high.

As it is seen in the Fig. 6, the activity-increasing effect of KCl is not impaired, i.e. a level of activity several times greater than the control is reached in the course of 10 minutes of examination, which even surpasses considerably the K^+-effect used as control. Hence it may be stated, that the effect of KCl is not inhibited but potentiated by TMAJ. This becomes especially evident when considering the number of contractions occurring during 10 minutes after the combined application of the two agents (TMAJ and KCl). Namely, in this

case, the number of contractions is greater than the sum of contractions caused separately by TMAJ and KCl under an equal period. With regard to the fact that TMAJ is not able even at high concentrations to produce lasting activity, it is suggested that the potentiation is due to the potentiated effect of K^+. The increase in time of the number of closed glochidia is the same in the presence of KCl and TMAJ as in the presence of KCl alone. In both cases about 20% of the glochidia are closed. This involves that potentiation is not due to

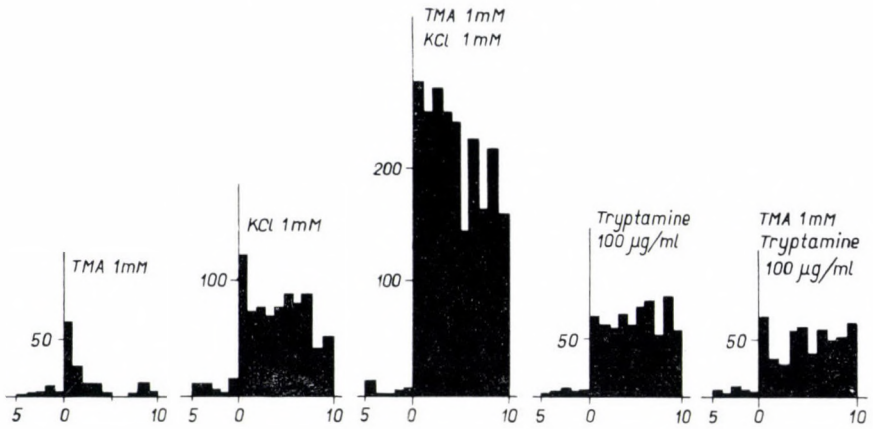

Fig. 6

the fact that there are in the presence of TMAJ many glochidia in open condition ready for producing rhythmic movement. Besides, synergism applies only to effects which are regulating rhythmic activity and to those producing tonic changes.

The activity increasing influence of 100 µg/ml tryptamine solution is inhibited to a small extent by 1 mM TMAJ. The number of contractions produced when using these two agents together amounts to 75% of that observed when using tryptamine alone.

Discussion

The results reported here furnish valuable informations in many respects. The one important question to be answered is in which way the agents that were and are still the objects of wide investigations in pharmacology as regards their effect on striated and smooth muscles of mammals and on various nervous structures, may influence the rhythmic activity of the embryonic smooth muscles of glochidia. It is worth mentioning that according to HERBERS [1] the glochidial smooth muscle is in that state of development, when the nervous

elements are still in the initial stage of differentiation and thus it is doubtful if they are innervated or not. When surveying the effects of the drugs used, it is striking that positive activity increase is observable only in the first minute after their addition. This common feature of every drug investigated leads to the assumption that the molecular receptors reacting to the drugs are either not differentiated or that the effect produced is not the symptom of specific tissue excitation.

When summing up the results, it is found that certain agents studied are capable of influencing in a considerable degree the activity-increasing effect of KCl and tryptamine. Atropine, nicotine, TEAC are most effectual in inhibiting K^+-effect. Hexamethonium is less effectual and curare has no influence. In the presence of TMAJ the potentiation of K^+-effect may be observed. The influence on the activity-increasing effect of tryptamine is somewhat different, as the inhibition caused by atropine and TEAC is less positive and nicotine proved to be completely ineffectual. Tryptamine effect is not or only to a small degree influenced by curare and hexamethonium. TMAJ, on the other hand, has a slightly inhibiting influence in contradiction to its potentiated influence on KCl-effect (Table 1).

Data are furnished by these studies also on the excitation processes of larval adductor. With regard to the fact that all drugs used are affecting cholinergic mechanisms, it is inferred that the effects induced by them under the conditions investigated may be explained by this property of the drugs. This means, that there are assumedly cholinergic mechanisms involved in the increased activity provoked by KCl, because this activity may most positive-

Table 1

The effect of the drugs investigated on the spontaneous glochidial activity produced by KCl and tryptamine

Drug	Effect on spontaneous activity	Influence on K^+-effect	Influence on tryptamine effect
TMAJ	Only initial activity-increase	Definite potentiation	Ineffectual
TEAC	Initially high, later low activity	Inhibition	Inhibition
Hexamethonium	Only initial activity-increase	Inhibition	Ineffectual
Nicotine	Only initial activity increase	Definite inhibition	Ineffectual or potentiated effect?
Atropine	Initially high, later low activity	Definite inhibition	Inhibition
Curare	Only initial activity-increase	Ineffectual	Inhibition

ly be inhibited by atropine, nicotine and TEAC. It is remarkable, that the inhibition of these substances on the activity-increase produced by tryptamine is not so effective. This may involve, that potassium and tryptamine are acting in two different ways. It is probable that tryptamine-effect is less dependent on cholinergic mechanisms than K^+-effect, i.e. it is completely independent from them, and may therefore be to a much lesser extent influenced by cholinotropic drugs. This may be connected with the assumption that there may be both cholinergic and tryptaminergic structures involved in the regulation of the action of embryonal adductor and that the effect of potassium may get realized through structures that may be paralyzed by cholinergic drugs.

The fact that potassium-effect is potentiated by TMAJ, whereas tryptamine-effect is inhibited by this agent, may similarly be explained by differences existing between the mechanisms of effect of potassium and tryptamine.

No final answer can be given on basis of these results as regards the origin of rhythmic activity. Nevertheless, it is a fact, that the "spontaneous" rhythmic activity is not eliminated when using the drugs investigated alone, which speaks against innervation or at least against its exclusive cholinergic character.

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DER EINFLUSS VERSCHIEDENER DROGEN AUF DIE RHYTHMISCHE AKTIVITÄT DER GLOCHIDIEN DER SÜSSWASSERMUSCHEL (ANODONTA CYGNEA L.)

Verfasser untersuchten die Wirkung verschiedener Drogen auf die spontane rhythmische motorische Aktivität der Larven Süßwassermuschel *(Anodonta cygnea* L.) ferner auf die durch KCl und Tryptamin produzierten erhöhten und dauernden Aktivitäten. Die Glochidien reagierten auf die meisten untersuchten Substanzen (Atropin, Hexamethonium, TEAC, Nikotin, Curare, TMAJ) mit einer nicht spezifischen anfänglichen Aktivitätserhöhung. Keine der untersuchten Agentien haben tonische Zusammenziehung verursacht. Atropin, TEAC und Nikotin haben die durch KCl gereizte Daueraktivität gehemmt. Man fand Hexamethonium in geringerem Maße hemmend und Curare war wirkungslos. Die Wirkung des K^+ war potenziert in der Anwesenheit von TMAJ. Der durch Tryptamin produzierte Einfluß wurde durch Atropin, TEAC, TMAJ und Curare in einem geringeren Maße gehemmt. Hexamethonium und Nikotin waren in dieser Hinsicht wirkungslos. Auf Grund dieser Ergebniße ist zu vermuten, daß zwei verschiedene Mechanismen bei der Wirkung der beiden Substanzen wie KCl und Tryptamin tätig sind, und ferner, daß die spontane rhythmische Aktivität von myogenischem Ursprung ist und auf der Natur des embryonischen Adduktors beruht.

ВЛИЯНИЕ РАЗЛИЧНЫХ ХОЛИНОТРОПИЧЕСКИХ АГЕНТОВ НА РИТМИЧЕСКУЮ АКТИВНОСТЬ ГЛОХИДИЕВ БЕЗЗУБКИ

Авторы исследовали влияние различных фармакологических веществ на фоновую ритмическую активность глохидиев беззубки и изучали эффект этих же агентов на вызванную хлористым калием и триптамином длительную ритмическую активность. Было установлено, что на большинство используемых фармакологических агентов (атропин, никотин, гексаметоний, кураре, ТМА, ТЕА) глохидии реагируют неспецифически, выражающиеся в начальном повышении их активности. Ни одного из изученных веществ не вызывало тонического закрывания глохидиев. Атропин, ТЕА и никотин вызывают явно выраженное торможение ритмической активности глохидиев вызванной хлористым калием. Менее выраженное торможение вызывал гексаметоний, а кураре оказался неэффективным. В наличии ТМА эффект хлористого калия был увеличен. Эффект триптамина блокировался с ТЕА, ТМА, кураре и атропином. Гескаметоний и никотин являлись неэффективными. Исходя из вышесказанных авторы предполагают, что хлористий калий и триптамин различным путем вызывают сильную и длительную ритмическую активность глохидиев. Предпологается тоже, что фоновая ритмическая активность глохидиев имеет миогенную а не нейрогенную природу.

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Acta Biol. Hung., **15** (2), 129—138 (1964)

THE EFFECT OF PREDNISOLONE (DELTA-1-HYDROCORTISONE) ON THE NEODIFFERENTIAL CARTILAGE FORMATION

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(Received November 15, 1963)

Synopsis

In 15 dogs semi-arthroplasty according to KROMPECHER and PUKY was performed and the animals were treated per os with prednisolone, 0.5 mg/kg body weight/day, over 70 days. Five dogs were not treated after operation, and served as control. After treatment, the animals were killed and the cartilage formed at the site of the joint surface cartilage removed was subjected to histological and carbohydrate-histochemical examinations. Even by gross inspection it was obvious that in the treated animals the articular surface layer was thinner than in the control. As further differences the blood vessels were usually smaller in diameter and vascularization was diminished by about 30 per cent, as compared with the control. In 6 animals of the 10 treated ones the area showing cartilage formation was greater in size than in the controls, but contained preponderantly younger cells (prechondroblasts). This chondrification took place in a fibrous environment, as opposed to the hyaline character found in the controls. The fibrous ground substance can be ascribed to the presence of younger forms of chondrogenesis producing comparatively less ground substance. As determined by Ritter—Oleson staining, in the treated cases the PAS positivity expanded at the expense of the Hale positive areas, but otherwise no significant difference from the controls was found. In four cases oligocellular, dense connective tissue containing collagen fibres was formed. The latter articular surfaces were even thinner than the average found in the prednisolone-treated animals. Thus, in response to prednisolone treatment, in most cases chondrogenesis increased in size, but was in some way inhibited in regard to the process of maturation.

Introduction

In the past decade, many researchers studied the problem of the hormonal influence upon tissue regeneration. The effects of the pituitary trophic hormones, of the sexual steroids, and mostly of the adrenal cortical hormones on the processes of proliferation and regeneration have been investigated.

It was found that mineralo- and glycocorticoids antagonize metabolic and regenerative processes alike [28]. The glycocorticoids inhibit the growth of proliferation tissue, and thus to this may be ascribed, at least in part, the beneficial effect they produce in proliferative conditions, for example in rheumatoid arthritis. According to the evidence published in literature, the destructions of cartilage, which have already developed, or which are developing, do not improve, on the contrary, in some cases a deterioration is demonstrable. As it is well known, the glycocorticoids inhibit mucopolysaccharide production [3].

On the basis of those outlined above it seems necessary to investigate how the glycocorticoids, notably prednisolone, would act on the special pro-

liferative tissue, which is produced at the site of the removed articular cartilage, and which furnishes the basis for the development of a hypoxybiotic tissue high in mucopolysaccharides: the articular cartilage.

Methods

In 15 dogs semi-arthroplasty was performed by the method of KROMPECHER and PUKY [16, 17] in the knee joint of the left hind leg. After exposing the knee joint, the femoral articular surface cartilage was sawn off together with a thin layer of spongiosa and a new articular surface was created, then the joint was closed.

Ten dogs were treated with 0.5 mg/kg body weight of prednisolone daily, for 70 days after operation. Five dogs served as control. After the treatment had been completed, the animals were killed, a predetermined part of the regenerating articular surface of the femur with the thin layer of spongiosa under it was removed, fixed in neutral formalin and Ca formol (1 : 9), then the specimens were subjected to the following treatments.

1. Staining with azan, staining with haematoxylin-eosin.
2. Staining according to *Ritter—Oleson* [24].
3. Acetylation + staining by the Ritter—Oleson technique (14, 24).
4. Acetylation + desacetylation + Ritter—Oleson staining, to determine the carbohydrate nature of the PAS positive substance.
5. Salivary digestion + Ritter—Oleson staining, to eliminate glycogen. Digestion was carried out at 37 °C for 2 hours.
6. Digestion with Hyase (Hyason, Organon, 150 U/ml, 16 hours, at 37° C) + Ritter—Oleson staining.
7. Digestion with pepsin (2 mg/ml of pepsin in 0.02 n HCl, 37°C, 5 hours) + digestion with trypsin (in a phosphate buffer of pH 8, 1 mg/ml, 37°C, 15 hours) + staining by the Ritter—Oleson method.
8. Staining with 0.1 per cent toluidine blue solution.
9. Methylene blue extinction staining at pH 1.42 [21, 22].
10. Digestion with pepsin + trypsin + methylene blue extinction at pH 1.42.
11. Hyase digestion + methylene blue extinction at pH 1.42.
12. Baker's phospholipid staining [14].

Results

It was evident by mere gross inspection that in most of the treated animals the articular surface layer was by about 40 per cent thinner than in the controls. The results obtained by the above histological and histochemical techniques may be described in brief as follows.

Fig. 1. Sagittal section of the newly formed femoral articular surface 70 days after the removal of the articular cartilage, showing the histological appearance of the fibrous cartilage which has developed after treatment with prednisolone, 0.5 mg/kg body weight /day. Groups of young chondrocytes are visible in between the collagen fibres, arranged at rectangles to the surface. H. E. staining. × 112

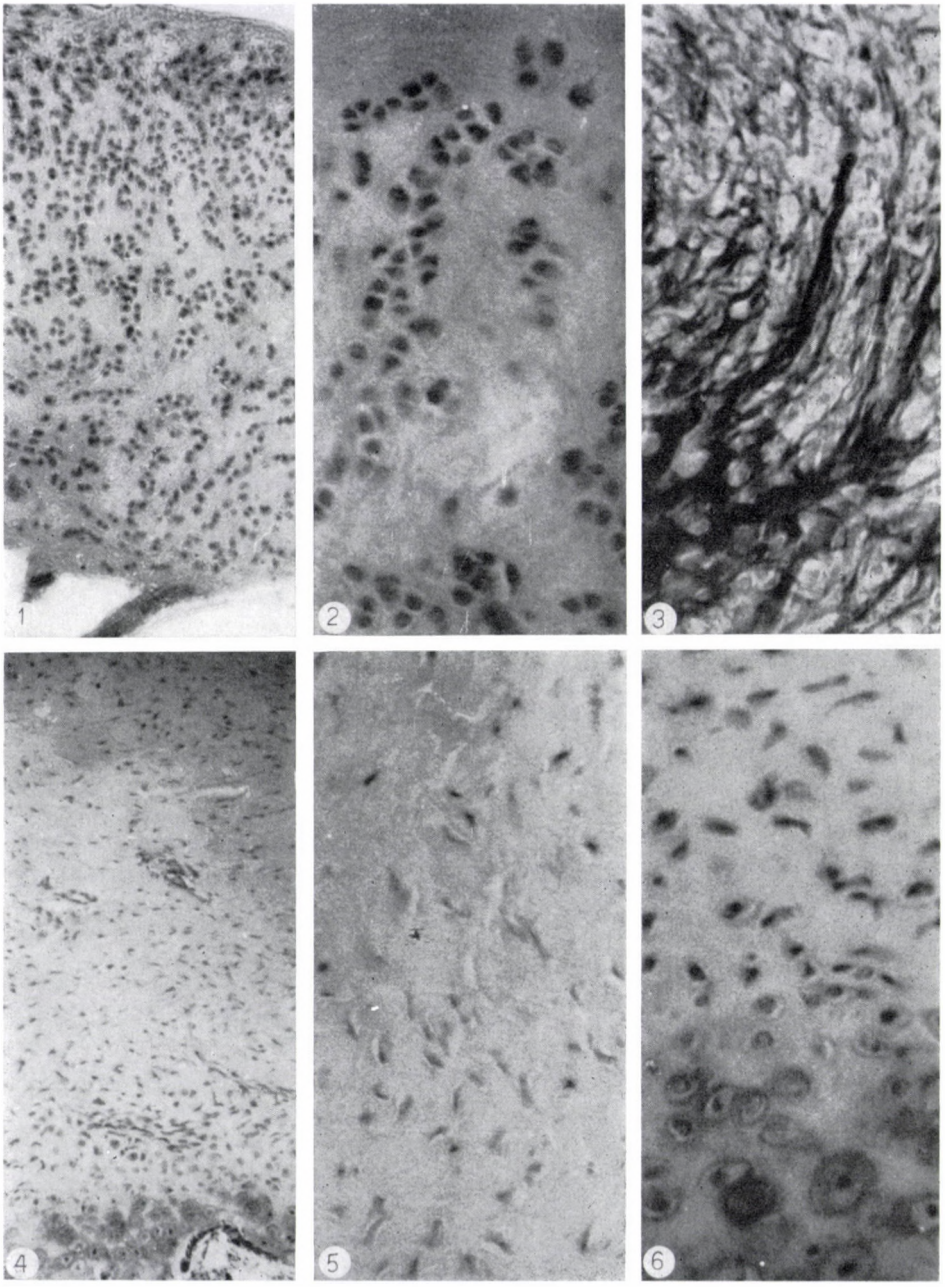
Fig. 2. High-power view of the uppermost layer shown in Fig. 1. As visible, encapsulated young chondrocytic forms are detectable even in this layer. × 310

Fig. 3. High-power view of the fibrous chondrogenesis in a prednisolone-treated animal at the articular surface-spongiosa junction. Azan stain. × 310

Fig. 4. Histological appearance of a section from a control animal, 70 days after removal of the articular cartilage. One islet of cartilage is visible in the lower one-third of the picture. H. E. staining, × 112

Fig. 5. High-power view of the uppermost layer of Fig. 4 showing granulocytic cells. × 310

Fig. 6. Control section, showing the high-power view of the articular surface-granulation tissue junction. An islet of cartilage is visible in the lower part of the picture. H. E. stain. × 310.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1. In the prednisolone-treated animals the histological pattern of chondrification diverged to some extent from the known pattern of normal neodifferential cartilage formation [9, 16, 17]. In the control sections cartilage formation starts in the form of islets of cartilage at the cartilage-spongiosa junction (Figs. 4 and 6) while in the treated cases it starts at the same place (Fig. 3), but the cartilage formed is more diffuse and is fibrous in character (Figs. 1, 2 and 3). In between the collagen fibres arranged at rectangles to the surface, groups of cells composed mainly of prechondroblasts and chondroblasts can be found.

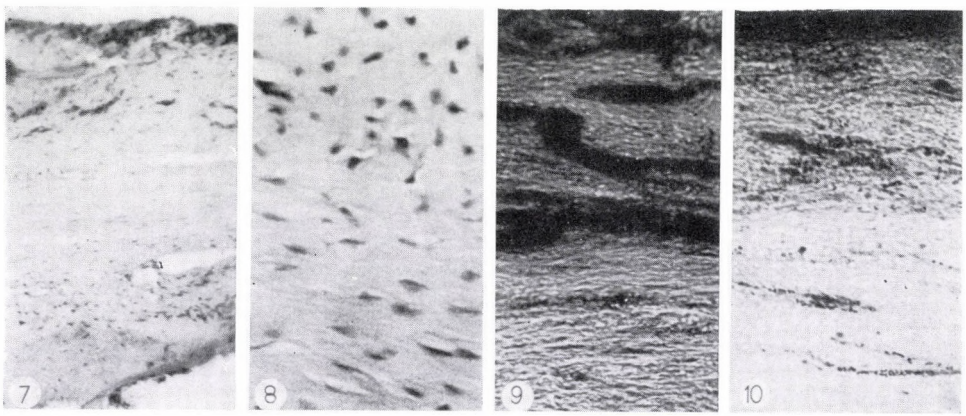

Fig. 7. Connective tissue of fibrous character, following prednisolone treatment. H. E. stain. ×112

Fig. 8. High-power view of the central layer of Fig. 7. ×310

Fig. 9. Sagittal section of the newly formed femoral articular cartilage, 70 days after the removal of the articular surface, showing the best vascularized granulation tissue area. The corpuscular elements of blood react well to Baker's phospholipid stain. Control section. ×112

Fig. 10. Sagittal section of the newly formed femoral articular surface, 70 days after the removal of the articular surface, following treatment with prednisolone 0.5 mg/kg body weight/day, from the area of the best vascularized granulation tissue. Baker's phospholipid staining. The blood vessels are diffusely narrower in diameter. ×112

In 3 cases this zone of chondrification makes up a higher proportion of the total articular surface layer than in the controls, and in 3 other cases it makes up the latter completely. In these latter 3 cases the staining of the ground substance is paler, and capsulated, young cartilage cell forms can be found even in the uppermost layer (Fig. 2), while in the controls this layer contains granulation tissue (Figs. 4 and 5). In 4 dogs the articular surface layer was even thinner than that, and under it dense collagen fibre connective tissue running parallel with the surface was found (Figs. 7 and 8).

There was also a difference in vascularization between the treated and untreated specimens. It is known that in the case of normal regenerative chondrification the proliferation tissue-spongiosa junction i.e. the area where chondri-

fication manifests itself in the form of islets of cartilage is the poorest in blood vessels; vascularization improves in the direction of the surface, and the superficial granulation tissue layer is the most richly capillarized [10, 19] (Fig. 9).

In the treated cases the blood vessels occurred diffusely in smaller numbers and were of smaller diameter (Fig. 10).

2. Following staining by the Ritter-Oleson method, there is a change in the proportions of the Hale and PAS positive areas, as compared with the controls.

In the control sections in the area of the islets of cartilage near the mature cartilage cells the ground substance is strongly PAS positive, whereas the ground substance of the layer containing chondroblasts and prechondroblasts shows Hale positivity. Thus, the PAS and Hale positive layers are alternating, parallel with the surface. (As to the description and problems of the carbohydrate histochemistry of neodifferential chondrogenesis see [11]).

In the four cases showing no chondrification the surface stained pale, but was almost exclusively PAS positive.

3. Acetylation + Ritter—Oleson. PAS positivity was practically absent.

4. Like in the case of the controls, PAS positivity recurred in the treated cases.

5. Following digestion with saliva, PAS positivity disappeared from the cartilage cells in both the control and the treated cases. The PAS positivity of the ground substance was unchanged following digestion with saliva.

6. Following digestion with hyase, no difference was demonstrable in the intensity of the Hale positivity, caused presumably by acid polysaccharides, by the Ritter-Oleson staining.

7. Following digestion with pepsin + trypsin the intensity and extent of the Ritter—Oleson staining were the same as in the undigested control and in the treated sections.

8. The metachromasia of the ground substance surrounding the mature cartilage cells was of an intensive beta type, while that around the prechondroblasts and chondroblasts showed gamma type metachromasia. In the treated cases the gamma type metachromasia appeared to more extensive in the above mentioned six cases, while in the 4 cases showing no chondrification merely a minimal gamma metachromasia was visible. Among the cells the chondroblasts and chondrocytes stained metachromatically.

9. It appears that in this case it is exclusively the acid mucopolysaccharides that possess methylene blue extinction at pH 1.42. The localization and type of the metachromatic areas were the same as in the case of staining with toluidine blue.

10. In both the treated and the control cases a minimal metachromatic reaction was detectable in the zone of mature chondrocytes.

11. In both the control and treated cases metachromatic staining disappeared following digestion with hyase.

12. Baker's phospholipid staining was employed as a supplementary procedure, to obtain information as to the extent to which the cells would contain phospholipids. The mature chondrocyte forms showed minimal reaction, the ground substance, like in the controls, remained negative. At the same time, this method of staining demonstrated excellently the differences in vascularization between the control and treated specimens (Figs. 9 and 10).

Discussion

In our experiments it has been found that in response to the systemic administration of prednisolone the articular surface layer became much thinner than it was in the controls. This is presumably ascribable to an inhibition of proliferation tissue formation at the site from which the articular cartilage has been removed.

Back in 1940, LOEB [23] reported that hormones might increase or reduce the rate of normal tissue maturation. The inhibitory action of cortisone upon the growth of granulation tissue was studied by HOWES and co-workers [13], and others [23]. These workers studied the time of development, the regularity or irregularity and vascularization of the callus following experimental fractures, its weight-bearing potency and found that following treatment with cortisone the callus developed late, was irregular and less weight-bearing [5, 7].

The action on granulation tissues induced by different methods has been extensively investigated [6, 23, 27, 29]. In tissue cultures it acted most intensively on the mesodermal elements, of the three primary germ layers. In fibroblastic cultures inhibition of growth and degeneration of cells have been observed [8]. The delay caused in wound healing might be attributed to its antiphlogistic effect and to its inhibitory effect on the formation of fibroblasts. This might be responsible also for the disturbance in collagenogenesis and ground substance formation.

In cortisone-treated chick embryos ROBERTS [25] found an increase in the free hydroxyproline concentration of the liver, heart and brain. It is likely that this amino acid cannot take part in collagenogenesis, like it can under normal conditions.

The reduced rate of ground substance formation is due presumably to an inhibition of mucopolysaccharide production [3].

LAYTON [20] has demonstrated that in response to cortisone treatment the sulphate content of the liver increases, while that of the heart and muscle decreases, which suggests that inorganic sulphate is not incorporated into chondroitin sulphate, an important mucopolysaccharide component of connective tissue, and especially of the ground substance of cartilage [12, 15, 26].

BOLLET [1, 2] has found a disturbance of glycosamine-6-phosphate synthesis in response to cortisone in the rat's liver and in the connective tissue grown in polyvinyl foam, and attributed this to a decrease of transaminase activity. Transaminase produces glycosamine-6-phosphate from glutamine and hexose-6-phosphate, that is an important component of mucopolysaccharides.

The fibrous chondrification observed in our experiments seems to be correlated with the reduced production of ground substance, and is not explained by an increased fibrogenesis. In the four cases already mentioned, in which no chondrogenesis could be demonstrated, the presence of the fibrous connective tissue is likewise due not to an increased fibre formation, but in the first place to a diminution of ground substance formation and probably to a diffuse diminution of capillarization. In these four cases several explanations may be put forward to explain the absence of chondrogenesis. In the first place, we should mention a reduction in, or an absence of, pressure on the articular surface, as a specific stimulus of the latter, but the age, regenerative potency and individual sensitivity to the hormone of the animals might also have been involved.

KROMPECHER and co-workers [9, 16, 17] have demonstrated that an absence of walking, as a pressure stimulus to which the articular surface is exposed, may influence chondrogenesis in the negative direction. Its primary importance is indicated also by the fact that chondrification takes place in those articular surface layers and at those points, which are exposed to the highest pressure, corresponding with the end-points of the spongiosa trabecules protruding into the granulation tissue.

KROMPECHER and TÓTH [19], as well as HADHÁZY [9] demonstrated in model experiments that also the occlusion of blood vessels and the reduction of vascularization were the most marked in that articular surface layer and at those points.

It is also known from preliminary investigations that in the course of regenerative chondrogenesis capillarization gradually deteriorates locally, and is in a cause-effect relationship with chondrogenesis [10].

The decrease by about 30 per cent of vascularization observed in the prednisolone-treated cases ought to have led to a relatively greater increase of mucopolysaccharides, as a result of the increased hypoxia of tissues [18], and ought to have increased chondrogenesis, but the disturbances of cellular metabolism and the resultant diminution of ground substance production presumably retarded the maturation of cartilage.

TAUBENHAUS and co-workers [29] have found that cortisone treatment reduced the number and inhibited the growth of capillaries in the granulation tissue of turpentine abscess.

In the 6 cases showing chondrification the extensive PAS positivity demonstrated by the Ritter-Oleson staining technique might be due to a sub-

stance of mucopolysaccharide nature, and might be a result of an increased rate of mucopolysaccharide breakdown. There have been no marked differences in the intensity of staining between the treated and the control specimens. Considering, however, that the surface was thinner, it may be assumed that the absolute mucopolysaccharide content has diminished, and together with it the amount of the ground substance also decreased. Investigations are under way to determine the actual quantitative relationships.

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DIE WIRKUNG VON PREDNISOLON (DELTA-1-HYDROKORTISON) AUF DIE KNORPELBILDUNG

An 15 Hunden wurden mit der Methode von Krompecher Halbgelenke gebildet. Nach dem Eingriff wurde den Tieren während 70 Tage per os Prednisolon verabreicht (0,5 mg/kg/Körpergewicht täglich). 5 Hunde dienten als Kontrolle und erhielten kein Prednisolon. Nach Beendigung der Behandlung wurden die Tiere getötet und die an der Stelle der entfernten Knorpelflächen entstandene neue Knorpelschicht wurde mit histologischen und kohlenhydrat-histochemischen Methoden untersucht. Bereits bei der makroskopischen Besichtigung war es auffallend, wie dünn die Gelenkflächenschicht der behandelten Tiere im Vergleich mit den Kontrolltieren ist. Bei den behandelten Tieren sind außerdem die Gefäße enger, und der Gefäßreichtum um etwa 30% geringer als bei den Kontrollen. Unter den 10 behandelten Tieren war das Gebiet der Knorpelbildung in 6 Fällen größer als bei den unbehandelten, enthielt aber überwiegend jüngere Zellen (Prechondroblasten). Die Knorpelbildung geht in diesem Fall in faserigem Milieu vor sich, im Gegensatz zum hyalinen Typ der Kontrollen. Die faserige Grundsubstanz läßt sich mit der Anwesenheit der jüngeren Knorpelbildungs-Formen erklären, diese bilden nämlich verhältnismäßig wenig Grundsubstanz. Bei Ritter—Oleson-Färbung ist das PAS-positive Gebiet gegenüber den Hale-positiven Gebieten ausgedehnter, sonst läßt sich aber im Vergleich zu den Kontrollen kein erheblicher Unterschied feststellen. In vier Fällen entstand ein zellarmes, dichtes Bindegewebe mit kollagenen Fasern. In diesen Fällen waren die Gelenkflächen noch dünner als im Durchschnitt der behandelten Tiere. Nach Prednisolonbehandlung kann demnach in der Mehrzahl der Fälle eine ausgedehntere, doch in ihrem normalen Reifeprozeß gleichsam gehemmte Knorpelbildung festgestellt werden.

ДЕЙСТВИЕ ПРЕДНИЗОЛОНА (ДЕЛЬТА-1-ГИДРОКОРТИЗОНА) НА НЕОДИФФЕРЕНЦИАЦИЮ ХРЯЩЕЙ

Авторы вызвали по методу *Кромпехера* у 15 собак образование полусуставов, и после вмешательства, животным вводили в течение 70 дней преднизолон в дозе 0,5 мг на кг веса тела в день. 5 собак служили контролем и не получили преднизолона. После прекращения дачи преднизолона животных убили, и возникший на месте удаленного хряща суставной поверхности новый хрящевой слой исследовали гистологическими и углеводно-гистохимическими методами. Уже при макроскопическом осмотре поражает тонкость слоя суставного хряща у обрабатывавшихся животных по сравнению с контролями. Кроме того, у животных, получивших преднизолон, сосуды были в общем более узкими и васкуляризация была около на 30% меньше, чем у контрольных собак. Среди 10 обрабатывавшихся собак в пяти случаях область хрящеобразования была большего размера, чем у контролей, но она содержала преимущественно более молодые клетки

(прехондробласты). Хрящеобразование происходит в этих случаях в волокнистой среде, в противоположность гиалиновому типу у контрольных животных. Волокнистое основное вещество объясняется присутствием более молодых форм хрящеобразования, которые образуют сравнительно меньше основного вещества. При помощи окрашивания по методу Риттер—Олесона у обрабатывавшихся животных наблюдается распространение ШИК-положительности за счет Гел-положительных областей; в остальном не удалось выявить значительной разницы, по сравнению с контролем. В четырех случаях была получена бедная клетками и плотная соединительная ткань с коллагенными волокнами. В этих случаях толщина суставной поверхности была даже меньше средней толщины хрящевого слоя у животных, получивших преднизолон. Следовательно, после дачи преднизолона в большинстве случаев наблюдается более распространенное хрящеобразование, однако, нормальный процесс созревания, по-видимому, является заторможенным.

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Acta Biol. Hung., **15** (2), 139—170 (1964)

CHANGE OF WEIGHT, LIPID AND WATER CONTENT OF LYMANTRIA DISPAR L. WITH SPECIAL REGARD TO THE CHEMICAL AND ENERGETIC CHANGES DURING INSECT METAMORPHOSIS AND IMAGINAL LIFE

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(Received November 26, 1963)

Synopsis

Author submits his investigations on the pupae and imagos of *Lymantria dispar* L. The results of studies on age, changes in weight, fat and water content testify on the specialized organism of the gipsy moth *(Lymantria dispar* L.*)*. This specialization derives from the fact that these insects do not feed in the imaginal stage, hence their food and energy supplies are covered by reserve substances stored during the larval stage. The reserve is utilized preponderantly to assure propagation. In the want of food uptake, they decompose the substances of their own bodies during life at a rate which calls, from the point of view of production biology, for the coining of a special term, defining these animals as self-decomposers. (The term is undoubtedly applicable to all other holometabolous insects which do not feed in the imaginal stage.) Though this characteristic is valid for both males and females, there is still a certain difference concerning the management of the reserves between individuals of the sexes, just as they differ also in morphological respects. Subsequently to the ceasing of food uptake by the caterpillars, the loss of weight of the males is so heavy that the weight of a dying moth is only 6.6 per cent of the greatest weight of the larva. The decrease of weight is further aggravated by the fact that the imago, after hatching from the pupa, frees itself from all dispensable material, so that its weight will diminish to the smallest possible amount which, again, results in its being able to convert a considerably lesser amount of energy covering the work of muscles involved in flight. The males thus utilize their available energy resources almost exclusively for flight in search of mates. The fat content of male moth is very high, 18.76% of the live weight at an age of 33 hours. This supply of fat will be gradually utilized during flight, although a fraction of it will still remain unused at the time of death of the moth. The water content also strongly decreases during the hatching from the pupa and in the first hours of the life of the imago. There is no more than 56.5% water found in a 24 hour old male moth. However, the relative water content will diminish but slightly or, in the second half of the period of flight, not at all. This is explicable by the fact that the insect utilizes the water produced in the oxydation of fatty substances. While the males use their food reserves and energy supplies mainly for flight, the females convert then the production of eggs. The placid, hardly moving females utilize far less material to cover their metabolic processes than do the males. If no eggs are laid, their weight would decrease only by 31.7% during the imaginal stage. Also their fat supply, relatively much less than that of the males, decreases but insignificantly. An oviposited female, on the other hand, presents quite another picture. The weight of such an insect at its death is generally but 19.2% of that after its hatching from the pupa. A considerable part of the fatty substances of the animal (about 80% of the initial supply) is also transmitted into the eggs. An oviposited female has on the other hand a higher water content than the eggs; this again proves that the insect transmits the substances of a higher energy content into the eggs.

Introduction

Concerning feeding and metabolism, holometabolic insects occupy a special place in the animal kingdom. They are primarily characterized by the suspension of food uptake during periods of metamorphosis. In the very metamorphic stage which involves extremely profound changes in their organisms, they are dependent solely on their reserve food substances. There is a special group of these insects: those which do not feed even during their imaginal stage. The special metabolic stage, characteristical of the pupae, extends in these insects to the end of their remaining life cycle. Such insects consume therefore their last aliment when still larvae, making subsequently preparation to pupae, often spin cocoons, pupate, metamorphose, copulate and oviposit as imagos, fly vividly, not infrequently covering distances of many kilometers, — all on their reserve substances and accumulated energy stores. The substance and energy management of these insects is so peculiar, their life in the ecological communities so significant, that they indisputably merit a special and detailed study.

Literature informs on a number of problems concerning the metabolism and the chemical processes occurring in the organism of the insect species in question. However, it is rather striking that imagos are far less known in this respect than the other developmental stages, albeit the special feature of the metabolism of insects not feeding in the imaginal stage are the most conspicious in just this latter phase. It was this very consideration which induced me to undertake the investigations set out below.

For the subject of study, I have selected the gipsy moth *(Lymantria dispar* L.), excellent in many respects for experimental purposes. I also paid due attention to the pronounced sexual dimorphism prevalent in this genus of moths. The sexually dimorphic imagos also essentially differ as to life habits. The males are extremely active animals flying for most of the time whereas the females are sedate and scarcely mobile. The females move so little that they lay their eggs generally in the vicinity of the pupa from which they emerged, and they do not fly at all. SCHEDL [37] is of the same opinion, and mentions a single case, citing FERNALD, when the moth made a short flight after oviposition. Hence the problem involving the difference between the males and females as to their utilization of the reserve substances is especially interesting.

In my study, I endeavoured to pursue a double project. On the one hand, I intend to submit the description and results of my investigations on the imagos and, in addition on some pupae of the species in question. The investigations bear on the duration of life of the imagos, the changes in their weight as well as the quantitative formation of their water and fat-lipoid content. I contend, namely, that the manner of management of the water and fatty

substances by the insects is, especially in their non-feeding periods, of decisive importance. On the other hand, I have tried to construe a survey of literature, compendious yet of a rather wider field, on some problems of the economy of substance and energy of the insects; in this review my own examinations are, as if imbedded, presented to the reader. Thus, while these latter might contribute to the former, literature will, by yielding a basis for comparison with my studies, afford a more general insight into our present information gained hitherto in this field of research.

Method

Investigations were conducted in summer 1956. The experimental specimens came from a Querceto-Potentilletum albae forest in the Buda hills. I collected the animals as pupae, except for some caterpillars, and placed them in the laboratory. The hatched females were transferred into smaller, part of the males into bigger, insectaria with meshed wire walls in frames. Some of the males, which were designed to be kept alive for a longer period, have been set free, after marking their wings, in the laboratory, thus affording them free movement approaching their natural condition. The animals have moved rather at ease in the laboratory, never flying at or beating against the windows.

The temperature of the laboratory varied between 24—27°C during the period of investigations.

For measurement purposes, the insects have been put into closed receptacles. Water content has been established by measuring the selected experimental animals first in the living state, then after a desiccation for 6 hours at 104°C. Though the measurement values represent desiccation losses, the amount of other, easily volatile components besides water is rather slight. Hence the rate of the loss of weight occurring during desiccation will, in the followings, be considered purely as a loss of water. Determination of the amount of fatty and lipoid substances was made by the Soxhlet extraction method. Prior to extraction, the animals were homogenized by Na_2SO_4. Petrolether was used as the extracting medium. (Therefore all fat content values indicated below represent the total lipoid content of the animals soluble in petrolether.) The period of extraction was 6—8 hours.

Results and discussion

Weight conditions during metamorphosis

Let us first consider the alterrations occurring in the weight of gipsy moths and holometabolic insects in general, subsequent to the discontinuance of feeding, that is, after the larva attains its maximum weight. Already at the beginning of this developmental stage, a rapid loss of weight occurs in the case of every insect. According to SCHEDL [37], the weight of the *Lymantria dispar* pupae is, at the beginning of the pupal stage, no more than 35%of that of the wholly developed caterpillar. For my part, I made only informatory examinations in this respect on *Lymantria dispar*, and found a less considerable loss of weight. However, the proportion between the weights of the two metamorphic stages was rather suggestive of that I observed in *Hyphantria cunea*, on a greater material [14]. In this latter insect, value in question was 52%. With the reservation therefore that for the final solution of the problem further

investigations are needed, I accept this latter value as such that indicates the pupal weight of the gipsy moth as related to the maximum larval weight.

During metamorphosis, an additional decrease in weight occurs in the pupa. As testified by literature data, the rate of decrease in weight considerably differs per species. According to ROEDER [35], the weight of the insects during the pupal stage is almost constant. In this respect, ROEDER refers to the studies, made on diverse species, of VANEY and MAIGNON, BIALASZEWICZ, DRILHON, et al., and connects the phenomenon with the insubstantial amount of water loss in the pupal stage. JÁNSKY [22] arrived at other results with respect to the house fly. He found namely that the pupae lose one-third of their weight, due to the very loss of water, during metamorphosis. (I shall later revert yet to the problem of water loss.) One should, however, take into consideration the fact that also the dry weight of the pupae of the house fly decreases not inconsiderably, and, as is well known for a number of other insects, to a greater degree in the inital and terminal phases of the pupal stage than in the medium one. JANDA and MAREK [20] found a loss of weight of 7—8% in the pupal stage of *Leucoma salicis*. JASIČ and MACKO [21] reported that the summer pupae of *Hyphantria cunea* lose weight in different rates, depending on the period of metamorphosis. If, within certain limits, the temperature is higher and metamorphosis quicker, the animals suffer a slighter loss of weight than at lower temperatures and during a longer period of metamorphosis. For instance, there was found a loss of 18.18% for a pupal period of 13 days, but 23.28% in the case of 19 days. Earlier [14], I have also studied the pupae of the fall webworm and established that the pupae had lost, by the end of their metamorphosis, about one fourth of their initial weight. The pupal period lasted 16 days in that case. The slight difference appearing in the results of the two experiments might, in my opinion, be explained by the possible difference in the relative humidity of the air surrounding the pupae. Concerning the changes in weight of the pupae of *Lymantria dispar*, I have but one or two measurement data of my own, still in need of supplementary evidence. Accordingly, these pupae also suffered a loss in weight of about 25% during metamorphosis. It shall be on this basis that I submit, in the followings, the rate of the change in the pupal weight.

As to the actual weight of the gipsy moth pupae, I obtained the following data: the weight of pupae immediately prior to hatching was, on the basis of 9 measurements per 8—10 specimens each, 232.8 mg (mean value; Table 3). The relevant literature presents ECKSTEIN's [10] work dealing also with the weight of *Lymantria dispar* pupae. Later MITHAT [30] reported important data. His experimental pupae had a weight of 305—650 mgs, much higher than those I have measured. This difference might derive from the well-known fluctuation in size of the gipsy moth. The weight of the female pupae is, according to MITHAT [30], about twice to thrice greater than that of the males, amounting

to 710—1850 grs. On the other hand, SCHEDL [37] found considerably smaller differences between the pupae of both sexes, the mean weight of the males being 1/3 to 1/2 less than that of the females. The size of the pupae depends also on temperature: heavier pupae are attained at 23—28°C than below 23°C.

The life span of imagos

The gipsy moths freshly hatched from the pupae are, as all other lepidopterous imagos, rather torpid at first. Meanwhile, the wings attain their natural expansion, and the animals discharge all superfluous decomposition substances originated during, and retained since, the pupal stage. My experimental male moths have excreted the major portion of these fluids during the first hours of their imaginal life, but not infrequently freed themselves from smaller portions only much later. The process usually terminated by the ninth hour of their lives, but, e.g., male gipsy moth No. 3 still excreted some decomposition materials when aged 24 hours (true, it was not too active until then). Subsequent to the discharge of the useless substances, flight began. The intensity of flight had usually rather increased for a while, then seemingly abated, but the animals flew, with some intervals of rest, until they were totally exhausted.

Table 1 presents data on the life span of some male imagos. It appears that the life span of copulated and uncopulated moths was nearly equal. It would seem therefore that copulation will not, or at least not conspicuously,

Table 1

The life span of male Lymantria dispar imagos

Copulated		Uncopulated	
male imagos			
Live weight at death (mg)	life span (hours)	Live weight at death (mg)	Life span (hours)
44.2	52	32.8	52
37.4	54	39.2	54
40.4	65	30.1	64
63.0	74	36.9	69
31.3	78	37.6	73
42.5	80	39.8	80
54.6	81	28.0	82
42.9	83	32.4	82
—	—	42.6	82
—	—	41.1	85
Average life span: 71 hours		Average life span: 72 hours	

influence the life period of the male gipsy moth. MITHAT [30] made similar observations. Nor is there any apparent interrelationship between weight and life span of the animals. In the conditions of the present experiments, the average life span of the male imagos was about 72 hours. According to ECKSTEIN [37], the males live 3—9 days. MITHAT [30] submits more detailed data. He found that an increase of temperature between 12—37°C shortens life, while higher relative aerial humidity lengthens it. The life span of the males is invariably 3 days at 24°C and 49—60% relative aerial humidity, and at 27°C and 70—75% aerial humidity, as inferable from his graph in this respect. These data therefore agree with my own experimental results.

The females live longer than the males. This observation might be accounted for by oviposition (the long time necessary for the process of egg-laying), and the adaptation to the preceding long wait for the males. As will be discussed later, the discharge of the decomposition materials retained from the pupal stage takes also longer in the females than in the males. The life span data of some copulated and uncopulated females are presented in Table 2. It is interesting to note that copulated females live apparently longer than those which had not mated. This is remarkable for the cause that literature often advances statements to the effect that the life of insect imagos is generally lengthened by the waiting for the execution of the reproductive functions. On the other hand, BALÁZS and BURG [2] have shown that, in the case of *Galleria mellonella*, copulation does not affect the life span of imagos.

The female gipsy moths discussed here lived, by the way, for a shorter period than those studied by ECKSTEIN [37]. This author contended namely that the female gipsy moths live for 8—13 days. This difference might also be explained by the divergence of the aerial humidity content and temperature, since it is known that the life span of the females is just as deeply influenced by both factors as that of the males. I may again refer to MITHAT [30] who found that the females live, for instance, for one day at 37°C and at a humidity of 49—60%, and for nearly 9 days at 12°C and at the same humidity — whereas at the same temperature and at a saturated humidity their life will be lengthened to 17 days.

Table 2

The life span of female Lymantria dispar imagos

Life span (hours) of females	Copulated	100	112	119	125	125	128	130	132	132	138	Average life span (hours)	124
	Uncopulated	99	103	106	115	115	117	120	123	129	—		114

The weight of imagos

In order to establish the characteristics of the changes occurring in the weight of the male imagos during their life, I have regularly measured the weights of four moths (Table 3). There is a definite similarity in the development of the weight of these four specimens. Their weight very considerably decreased in the first 9 hours. This is the period of the discharge of decomposition substances. Further on, the animals lost gradually less and less, but the rate of the decrease of their weight remained largely uniform throughout.

Table 3

The formation of the live weight of some Lymantria dispar male imagos

Moth 1			Moth 2			Moth 3			Moth 4		
Age (hour)	Live weight (mg)	Per cent weight of imago as related to weight at hat hing	Age (hour)	Live weight (mg)	Per cent weight of imago as related to weight at hatching	Age (hour)	Live weight (mg)	Per cent weight of imago as related to weight at hatching	Age (hour)	Live weight (mg)	Per cent weight of imago as related to weight at hatching
0	139.7	100.0	0	145.2	100.0	0	151.4	100.0	0	155.1	100.0
4	87.2	62.4	4	86.0	59.2	9	77.9	51.5	9	78.6	50.7
9	65.3	46.7	9	69.7	48.0	24	71.3	47.1	24	72.5	46.7
15	63.2	45.2	15	67.1	46.2	34	65.1	43.0	34	64.0	41.3
24	55.3	39.6	24	64.5	44.4	48	61.0	40.3	48	58.5	37.7
38	50.8	36.4	38	53.9	37.1	58	53.8	35.5	58	50.1	32.3
48	43.6	31.2	48	45.5	31.3	72	47.1	31.1	72	45.8	29.5
62	39.4	28.2	62	40.1	27.6	82	42.6	28.1	85	41.1	26.5
69	36.9	26.4	69	38.4	26.4						
			73	37.6	25.9						

The weight data of the regularly measured imagos as well as other measurement values made it possible to construct a general formula expressing the changes in the weight of the males. In my calculations, I relied on the mean values of the proportions for the weights measured at different age phases of the moths presented in Table 3. As a starting-point, I established the average weight of the animals at the time of their death. This was found to be 39.6 mg on the basis of the data shown in Tables 1 and 3, and those of the moths used for obtaining the water and fat contents.

Working backwards from this point, I calculated the mean weights, by referring to the proportions mentioned above, of the specimens at their different age levels. The results of the calculations, together with the average pupal weight and the proportions of the weights of the moths at different age levels as related to their weights at hatching, are submitted in Table 4. The data

of Table 4 were compared with certain other direct measurement values, and I found a close similarity between the two. According to Table 4, the weight of the male moths was 148.3 mg at hatching, and the mean weight of 58 specimens measured at the same age was found to be 153.4 mg.

On the basis of the weight data now at our disposal we can establish, as the most striking phenomenon of all, that the loss of weight of the animals is remarkably high after the cessation of feeding. This is shown vividly in Table 5, which displays the ratios of the weight of the several developmental stages as related to that of the completely developed larva. As is to be seen, the weight of the animals decreases to half its value at the beginning of the

Table 4

The formation of the live weight of a male Lymantria dispar of average size

Live weight of pupa before hatching of imago	Live weight of moth			
	At hatching	4 hours age	9 hours age	72 hours age (at time of death)
232.8 mg	148.3 mg 100.0%	90.2 mg 60.8%	73.0 mg 49.2%	39.6 mg 26.7%

Table 5

The live weight of a Lymantria dispar male in various developmental stages, as compared to the maximum weight of the caterpillar

Maximum weight of caterpillar	Pupa at pupation	Pupa before hatching of imago	Imago at hatching	Imago at death
100%	52%	39%	25%	6.6%

pupal stage. The subsequent loss of weight (apart from the known loss of water during the pupal stage) testifies on the utilization of a high amount of energy indispensable for metamorphosis. During the hatching of the male moth, there will occur a further loss of weight, more than 36%, as related to the pupal weight. This finding again substantiates what was said above, namely that the loss of weight arises in this phase, besides of that incurred by the casting away of the exuviae, from the discharge of the decomposition substances formed during the pupal stage. This process terminates in the first few hours of the life of the male imagos, effecting a further loss of weight (39.2% in 4 hours as related to the initial weight of the animal, and 50.8% in 9 hours; (Table 4). The rate of the loss of weight is further increased by the removal of

all dispensable materials from the organism of the moth. Its weight will decrease to the smallest possible amount, which affords a highly economical utilization of the reserve energy substances during flight. We may safely assert that, when the time for flying arrives, the organism of the male animal has already been modified to the extreme for the exigencies of flight and of the reproductive act. Any other, largely uniform, loss of weight during the period of flight derives practically from flight alone, caused, besides the slight loss due to the wearing of the external chitinous cover, by oxidation processes occurring during flight and the loss of water (see below). Ultimately, it is well-nigh astounding to find, when considering these data, this drastic loss of weight, according to which the weight of an old moth is not more than one quarter of that of the young imago, and only 6.6% of that of the assumed maximum weight of the larva.

The change of weight of the female moths was demonstrated, similarly to that of the males, by repeatedly weighing some selected individuals in the course of their imaginal life. Table 6 presents the weighing data of four uncopulated females. It appears, first of all — a fact also observed on a greater material — that uncopulated females begin ovipositing towards the end of their life. They lay usually only a fraction of their eggs, sometimes a greater quota. This pile of eggs, laid as if by compulsion, differs essentially from the regular cluster of eggs produced by copulated females. Uncopulated females lay their eggs in an irregular fashion; the eggs are usually not covered by abdominal hairs, and frequently scattered around. FERNALD [37] is perfectly right in stating that the two kinds of egg clusters can be easily distinguished with some practice. With respect to Table 6, it is to be noted that the weight of the first eggs was not again established at the time of the second weighing of the females, if they failed to lay fresh eggs in the meantime. In such cases therefore Table 6 shows, with respect to the eggs, the preceding measurement value. Although during this intervening period the weight of the eggs, too, will decrease, I deemed the inaccuracies arising of this omission as negligible owing to its very insignificance. The last two columns of Table 6 show hypothetical weights which would hold if the animals had not oviposited at all.

Prior to discussing Table 6 any further, I have to call attention to Table 7 which shows, in three phases, the general formation of the weights of uncopulated females and their eggs laid. To establish the average weight of the young females, I have utilized the corresponding data of Tables 6 and 8, and also the weights of five other females weighed at their hatching from the pupae, to wit: 349.9; 369.6; 398,6; 456.3; 461.1 mg. All other weight data have been related to the average weight of the young females, with respect to the general character of the changes in weight of the females and their eggs presented in Table 6. (The age of 114 hours designates, in the terms of Table 2, the date of death of the specimens.)

Table 6

The formation of the live weight of some uncopulated Lymantria dispar females and their egg-clusters

Age (hours)	Live weight of imago (mg)	Per cent live weight of imago as related to weight at hatching	Live weight of oviposited eggs (mg)	Per cent live weight of oviposited eggs as related to live weight of imago at hatching	Combined live weight of imago and its eggs (mg)	Per cent live weight (combined) of imago and its eggs as related to live weight of imago at hatching
			Moth 1			
0	477.6	100.0	—	—	477.6	100.0
24	384.7	80.5	—	—	384.7	80.5
48	369.1	77.3	—	—	369.1	77.3
71	356.1	74.6	—	—	356.1	74.6
103	339.8	71.1	3.2	0.7	343.0	71.8
122	316.7	66.3	13.5	2.8	330.2	69.1
129	311.0	65.1	13.5	2.8	324.5	67.9
			Moth 2			
0	345.4	100.0	—	—	345.4	100.0
24	267.1	77.3	—	—	267.1	77.3
48	253.8	73.5	—	—	253.8	73.5
71	230.8	66.8	10.4	3.0	241.2	69.8
103	138.7	40.2	81.0	23.4	219.7	63.6
			Moth 3			
0	358.4	100.0	—	—	358.4	100.0
24	294.4	82.1	—	—	294.4	82.1
48	281.3	78.5	—	—	281.3	78.5
71	274.1	76.5	—	—	274.1	76.5
103	260.7	72.7	—	—	260.7	72.7
120	247.3	69.0	8.4	2.3	255.7	71.3
			Moth 4			
0	451.2	100.0	—	—	451.2	100.0
24	375.9	83.3	—	—	375.9	83.3
48	361.5	80.1	—	—	361.5	80.1
71	347.8	77.1	—	—	347.8	77.1
103	314.9	69.8	10.7	2.4	325.6	72.2
117	306.9	68.0	10.8	2.4	317.7	70.4

The data of Tables 6 and 7 imply the following: the average weight of the female gipsy moths at the time of their hatching (402.7 mg) is slightly more than 2.7 times greater than the weight of the males of the corresponding age (148.3 mg). This difference in weight agrees, essentially, with MITHAT's [30] findings concerning the pupae of the two sexes of the gipsy moth.

The decrease in weight of the females is more intense initially than later, the same as with the males. This is caused also here by the discharge of the decomposition materials accumulated in the pupal stage. However, this process is more protraced in the females, owing to their longer span of life, and it involves, according to my observations, about 24 hours. On the other hand, the decrease in weight is by no means as considerable as in the males. While, namely, the

Table 7

The formation of the live weight of an uncopulated, non-oviposited female Lymantria dispar imago, of average weight

	Imago		
	at hatching	24 hours age	114 hours age
Live weight of imago	402.7 mg 100.0%	325.4 mg 80.8%	247.1 mg 61.4 %
Live weight of oviposited eggs	—	—	27.9 mg 6.9%
Combined live weight	402.7 mg 100.0%	325.4 mg 80.8%	275.0 mg 68.3%

weight of the males decreases by almost half during the first 9 hours, the females lose slightly less than 20% of their total weight in 24 hours. The difference might probably be explained by the assumption that the female imagos do not free themselves as radically of all superfluous substances as do the males, indeed, since the former do not fly, it is not at all important for them to have the lowest weight possible. Later, in the hypothetical case of never laying eggs, they will lose weight to a largely uniform ratio until their death. However, their decrease in weight is more moderate also after the first 24 hours than in the case of the males. These latter weigh no more at the time of their death than about 26.7% of their weight at hatching, while the former, provided they do not oviposit, decrease but to 68.3%. Owing to their torpidity and limited movements, the females therefore consume much less energy (oxidize a smaller amount of substances) than do the males.

The changes in the weight of some copulated females are shown in Table 8. These specimens mated in the first 15—20 hours of their lives. They differed insofar from the uncopulated females that, first of all, they began to oviposit

Table 8

The formation of the live weight of some Lymantria dispar females, of average size, and their egg-clusters

Age (hours)	Live weight of imago (mg)	Per cent live weight of imago as related to weight at hatching	Live weight of oviposited eggs (mg)	Per cent live weight of oviposited eggs as related to live weight of imago at hatching	Combined live weight of imago and its eggs (mg)	Per cent live weight (combined) of imago and its eggs as related to live weight of imago at hatching
			Moth 1			
0	351.1	100.0	—	—	351.1	100.0
20	283.7	80.8	—	—	283.7	80.8
77	76.2	21.7	181.3	51.6	257.5	73.3
100	73.3	20.9	181.3	51.6	254.6	72.5
			Moth 2			
0	431.4	100.0	—	—	431.4	100.0
20	340.5	78.9	—	—	340.5	78.9
77	114.4	26.5	198.0	45.9	312.4	72.4
100	77.0	17.8	216.4	50.2	293.4	68.0
120	74.3	17.2	216.4	50.2	290.7	67.4
125	73.2	17.0	216.4	50.2	289.6	67.2
			Moth 3			
0	418.3	100.0	—	—	418.3	100.0
20	339.5	81.2	—	—	339.5	81.2
77	127.9	30.6	167.0	39.9	294.9	70.5
100	99.3	23.7	183.1	43.8	282.4	67.5
120	69.4	16.6	199.8	47.8	269.2	64.4
138	66.5	15.9	199.8	47 8	266.3	63.7
			Moth 4			
0	365.2	100.0	—	—	365.2	100.0
20	296.8	81.3	—	—	296.8	81.3
77	118.1	32.3	159.0	43.5	277.1	75.8
100	94.4	25.8	170.1	46.6	264.5	72.4
120	72.9	20.0	181.5	49.7	254.4	69.7
132	70.4	19.3	181.5	49.7	251.9	69.0
			Moth 5			
0	404.3	100.0	—	—	404.3	100.0
20	331.8	82.1	—	—	331.8	82.1
77	115.8	28.6	177.4	43.9	293.2	72.5
100	92.6	22.9	187.2	46.3	279.8	69.2
120	88.5	21.9	187.2	46.3	275.7	68.2
130	86.9	21.5	187.2	46.3	274.1	67.8

much earlier, within 24 hours after copulation. They laid the eggs regularly and completely. In the course of oviposition, the animals laid the major portion of their eggs on the first day and the rest rather sluggishly in the succeeding one or two days. I have observed this also in the case of many other females not presented in Table 8, and SCHEDL [37] reported the same ovipositing pattern in the species.

Concerning the changes in the weight of a copulated female during its life, and the weight of its eggs, we have to refer to Table 9. The data of Table 9 have been computed as follows. On the basis of Table 2, I regarded the average life span of a female as 124 hours. Now, the end of the ovipositing period occurs, according to Table 8, generally 22 hours before the death of the insect. However, I have found according to observations made on 18 other females, that a period of 24 hours between the cessation of ovipositing and the animal's death is even more frequent. So, I have used this datum in Table 9. The weight given for the time of hatching from the pupa is taken as identical with the corresponding value of Table 7. I have calculated the weight of a 20 hour old animal — in the case of the moths presented in Table 8 — according to the ratio of the average decrease in weight up to this age. In establishing the weights

Table 9

The formation of the live weight of a copulated, oviposited female Lymantria dispar imago of average size, and its egg-cluster

	Imago			
	at hatching	20 hours age	100 hours age (termination of oviposition)	124 hours age (death of imago)
Live weight of imago	402.7 mg (100.0%)	325.8 mg (80.9%)	80.9 mg (20.1%)	77.3 mg (19.2%)
Live weight of oviposited eggs	—	—	195.8 mg (48.6%)	195.8 mg (48.6%)
Live weights combined	402.7 mg (100.0%)	325.8 mg (80.9%)	276.7 mg (68.7%)	273.1 mg (67.8%)

of a 100 hours old moth and its eggs laid, I have again referred to the changes in weight of the moths appearing in Table 8, and the weight of their eggs. Accordingly, the average live weight at the cessation of oviposition of the five females is 19.8% of that at the time of their hatching from the pupae. The weight of the eggs of these same animals is 49.1% of that of the female measured at the time of its hatching. I have also taken into account the corresponding results of the weighing of five other moths. These measurements gave the following values: 15.8, 19.7, 21.3, 21.9, 23.6% for the imagos, and 45.4, 46.9, 48.2, 48.7, 51.2% for the eggs. Finally, I have computed the weight of the

animal at the time of its death on the basis of the fact that the average weight of the moths presented in Table 8 had further decreased by 4.5% subsequent to oviposition as related to the weight at the end oviposition. I consider the weight of the eggs also in this case as unchanged after the termination of ovipositing.

A comparison of the data of Tables 7 and 9 reveals that there is no essential difference in the developments of the total weights (moth and its eggs) of the copulated and uncopulated females. It seems therefore that the process of ovipositing does not require any greater amount of energy. That the females still show signs of utter exhaustion after oviposition implies that there is only a small amount of reserve energy convertible for the requirements of the animal proper (not destined for the eggs).

The weight ratios of the oviposited females and the eggs laid by them are worthy of note. The fact that the weight of the eggs is nearly two and a half times of that of the females proves that the entire organism of the animal had been extensively adapted for the purposes of oviposition. The animal doubtless accomplishes the maximum performance by the production of this amount of eggs.

Chemical changes during metamorphosis and in the imaginal stage

Glycogen

As the amount of the loss of weight incurred during the pupal stage can be highly different per species or species-groups, similarly there are observable differences in the course of metamorphosis also with respect to the utilization of the reserve substances. In the honey-bee, the energy requirements of metamorphosis are primarily covered by glycogen. This is apparent already from the high glycogen content of the mature bee larvae. According to PFLUGFELDER [33], glycogen makes up, in this stage, one-third of the dry weight; while ROEDER [35] puts it at 32.5% for the larvae of the workers, and 25.5% for those of the drones. STRAUS [41] also showed it experimentally that it is glycogen which is primarily utilized in the pupae of bees, so that the glycogen content of the pupae decreases from the initial 6.2% to 0.5%, as related to the live weight. True, the fat content will also significantly decrease in the same period. SNODGRASS [40] also remarks that the bee pupae exhaust almost completely their glycogen reserves until they are ready to hatch.

At the time of metamorphosis, *Gastrophilus* also disposes of a considerable glycogen reserve. According to KEMNITZ, the mature larvae contain 31% glycogen [25]. UVAROV [49] writes about 14—31% glycogen in these animals. Glycogen, as a stored nutritive material, might have an important role also in the metamorphosis of *Calliphora erythrocephala*, since FREW [13] observed that no fat is utilized in the organism of these insects in the initial phases of the pupal stage. I venture to state, on the contrary, that there is a fat synthesis

in this period, as was shown for some higher flies to be discussed later.

In the larve of *Prodemia*, as reported by BABERS [1], glycogen appears already at the beginning of feeding; its importance during metamorphosis is satisfactorily proved by the fact that the percental participation of glycogen attains its maximum in the initial phases of pupation, to decrease in the pupa, and again increase in the imago. ROCKSTEIN [34] contends that several insects utilize 73—100% of their reserve glycogen content during metamorphosis.

Glycogen is of especial importance during hibernation. USHATINSKAYA [46] reports that the conversion of glycogen is possible also at a low temperature in invertebrates. The observation that summer pupae of the second generation of *Hyphantria cunea* utilizes mainly fatty substances, and the hibernating one primarily glycogen, is probably referable to the above statement. At least, we might infer as much from concordant observations of SZALAY—MARZSÓ [42] and JASIČ et MACKO [21] that the fat content of the summer pupae is higher than that of the wintering ones.

It is also worthy of note that a number of insects show differences as to sex, concerning the amount of stored glycogen, already in the larval and pupal stages. In such cases, females contain more glycogen than the males, which latter have a higher fat content. *Prodemia* illustrates this difference convincingly [1]. But we shall yet return to this problem in the discussion of the fat content of insects.

Informations are rather meagre on the glycogen reserves and their utilization in imagos. There is no doubt, however, that glycogen has an important role also in this stage in the metabolism of insects. May I again refer to investigations made on *Prodemia* [1]. In this animal the glycogen content after diminishing in the pupal stage, increases again in the imago. — The differences as to sexes, mentioned above with respect to the fat and glycogen content apparent already in the pupae, also proves that the latter substance is more important for the females than the males. It is quite certain that this phenomenon is interrelated with reproduction and oviposition. — In supplying energy for the flight of the vagile male insects, of especial interest for us, glycogen plays but a secondary role as compared to the fatty substances. However, there are exceptions known also in this respect, e.g. *Drosophila*. WIGGLESWORTH [53] kept adult vinegar flies without food at 25°C, and they had wholly utilized also their glycogen reserves, beside the fatty substances, in their activities during 48 hours. According to WIGGLESWORTH, only glycogen can be mobilized with the rapidity to cover the enormous energy requirements of the indirect flying muscles of *Drosophila*. Energy is directly derived from glucose. Entirely exhausted *Drosophila* flies can be induced to flight after feeding them glucose [55]. According to CHADWICK and GILMOUR [7], CHADWICK [6], WILLIAMS, BARNESS and SAWYER [57], *Drosphila* utilizes exclusively carbohydrates during flight.

Protein

Proteins play an indubitably minor role in the energy supplies during the metamorphosis of holometabolic insects. Still, one ought to take note of JÁNSKY's [22] assertion that there is a certain amount of protein breakdown in this period. LUDWIG [29] reports that the lepidoptereous pupae have a 1550 mg% protein-nitrogen content ("Eiweiss—N—Gehalt") which decrease to 390 mg% prior to the hatching of the imago.

Fat — lipoid substances

At our present state of information it seems that the most important energy source of insect pupae are fatty substances in the majority of cases. For example, TANGL [43, 44] had shown that, for *Ophyra cadaverina*, more than 90% of the utilized substances are fats. ROEDER [35] reported that *Galleria mellonella* covers its sugar requirements exclusively from fats and proteins, since this animal does not store glycogen at all.

Hyphantria cunea just as unable to feed as the gipsy moth, intensively stores fats in the last larval instar. In this period, its fat content increases by 4.2% as related to the 1.3—2.0% of the living weight of the previous instar [4]; concurrently, the energy content per gram of their dry body substances from 5200—5300 calories to 5900 cal [16]. Finally, the fat content reaches 8.8% in the fresh pupa [14]. In developed *Bombyx mori* larvae, FARKAS [12] found 7.1 dry weight % fat. YAMAFUJI, [58], NIEMIERKO, WLODAUER and WOJTCZAK [32] showed an even greater amount of fat-substances in the silkworm. It seems that, on the fifth day of the last instar, the fat content of the *Bombyx mori* larvae may even attain 16.46 dry weight %. According to PFLUGFELDER [33], the fat content might increase to 45% of the dry weight in the larvae of certain holometabolic insects. Later, however, the amount of fat will strongly decrease in the pupae, due to metamorphic processes. In *Hyphantria* pupae, about half of the fat reserves will be utilized [14]. According to the reports of WEINLAND [51], RUDOLFS [36], BECKER [5], KOZHANTSHIKOV [23], JÁNSKY [22] PFLUGFELDER [33], it seems that at least half, but usually more (varying per species) of the original fat reserve is decomposed in the pupa. Lepidoptera with not-feeding imagos belong among those species which utilize relatively small amounts of fat in the pupal stage. The decrease of the fatty substances is presumably of a like (smaller) ratio in the pupae of the gipsy moth, though I have no data in this respect. I have ascertained, however, that the male pupae contain 3.98% fatty substances (Table 10) before hatching.

The paramount role of the fatty substances during metamorphosis is substantiated by another observation, namely that some insects at least, synthesize fats during the pupal stage, converting other reserve substances for

this purpose. DITMAN and WEILAND [9], YEAGER and MUNSON [59], MUNSON and YEAGER [31] claim that there is an observable connection between the increase of the fat content and the decrease of the glycogen reserve in the pupal stage. In the pupae of *Lucilia sericata*, EVANS [11] showed fat synthesis. The fat reserves stored during the larval stage of this insect vanish by the end of the same stage, and the amount of fat necessary for metamorphosis is synthesized in the initial pupal phase. LEVENBOOK [27] has also shown in the case of *Gastrophilus intestinalis* that the animal disposes of the maximum fat content only some days after pupation.

Table 10

The lipid content of the male Lymantria dispar

Designation of age	Number of specimens examined	Lipid content (per cent of live weight)	Amount of lipid in specimen of average weight (mg)
Pupa before hatching of imago	10	3.84	
Idem	10	4.12	
Mean value		3.98	9.3
Imago at hatching	10	6.84	10.1
Imago 3 hour age	8	13.03	
Imago 4 hour age	8	11.58	
Idem	10	12.64	
Mean value		12.11	10.9
Imago 24 hour age	8	18.10	11.0
Imago 33 hour age	15	18.76	10.8
Imago 70—80 hour age (at death)	16	10.91	
Idem	10	9.87	
Mean value		10.39	4.1

The importance of the fats in the period of metamorphosis lies doubtless in the fact that they have a higher energy content than either the carbohydrates or the proteins, hence they can more suitably cover the energy requirement in non-feeding organisms. If this be so, it is only to be expected, that the fat content of insects will eventually increase previously to other non-feeding periods, too. And in fact USHATINSKAYA found that in *Cydia pomonella* the fat content increases from 11 to 18% prior to the diapause [33]. During its diapause, *Leptinotarsa decemlineata* draws mainly on fats apart carbohydrates, as sources of energy [48].

It seems that the decrease of fatty substances is not uniform during the pupal stage. As, among others, JÁNSKY [22] has shown on *Musca domestica*, and GERE [14] on *Hyphantria cunea*, the amount of fat diminishes rapidly in the initial and terminal phases and essentially slower in the median phase of the pupal stage. According to LEVINSON and SILVERMAN [28], 400 μg of the total lipoid reserve (1225 μg) of the pupa of *Musca vicina* is utilized in the early pupal stage, and another 400 μg prior to the hatching of the imago. These findings are in complete harmony with WIGGLESWORTH's [54] reports. The combustion of fats is a process involving a high amount of oxygen, and in the median phasis of the pupal stage, when hystolysis is the most pronounced, the oxygen supply is reduced. In this period the necessary energy is supplied largely by glycogen instead of fats.

The fat content of the pupae differs depending on sex. This is especially conspicuous in the species which cover their imaginal energy requirements from reserve substances stored in the larval stage, and the more so if the imagos of both sexes have diverse habits and locomotive activities. Good examples in this respect are the pupae of *Bombyx mori* [50, 58, 32], *Hyphantria cunea* [42], and *Leucoma salicis* [20]. The male pupae invariably excel by a higher fat content, thus providing for the energy requirements of the imaginal life. We shall see later that the male moths, considerably more active than the females, have especially high requirements for fatty substances rich in energy. These organisms endeavour to fulfil their claims to the utmost in this respect. It was shown for example on *Leucoma salicis* that in the male pupae, besides their higher fat content mentioned above, the reserve of fatty substances diminish slower during metamorphosis than in the females [20]. The fat content of the females decreased during the pupal stage from 11.4% to 7.5% while that of the males from 15.1% to 12.6% as related to the initial weight of the pupae.

Great though the differences be concerning the quality and quantity of the reserve substances during metamorphosis, there is a certain similarity, as we have seen, in this respect among the lepidopterous species with non-feeding imagos. They agree for instance, apart from the striking difference displayed in the pupal fat content depending on sex, in that their fat reserves diminish, though moderately yet still definitely, during metamorphosis. *Lymantria dispar* also belongs to this group. In what manner does the amount of fatty substances change in the males of this species? The answers to this problem are presented by the data of my investigations shown in Table 10. These data indicate that the per cent fat content as related to the live weight of the moths increases extraordinarily during the hatching from the pupae and the initial phase of the imaginal life. The relative increase in the fat content coincides with the abrupt decrease of the weight of the animals. We have thus concrete proofs of the assumption that, in this period, the males free

themselves of valueless, energypoor substances; they become lighter and thus need a slightly smaller amount of energy to meet the requirements of the enormous muscular work involved in flight. The 18.76 live weight % of fat content an enormous proportion in the 33 hour old male moth implies that the whole energy required for flight is covered practically by the fats alone.

However, it is not only the gipsy moth males which dispose of a similarly high fat content. Literature submits like records concerning other non-feeding lepidopterous imagos. JANDA and MAREK [20] state, with respect to *Leucoma salicis*, that of the several developmental stages of the males and females the fat content is especially high in the male imagos. GILBERT and SCHNEIDERMANN [18] state referring to NIEMIERKO's and YAMAFUJI's investigations on *Bombyx mori*, that the young male moths have the greatest amount of fat. BALÁZS, KOVÁTS and BURG [3] report on similar observations on *Galleria mellonella*, a species systematically distant yet also with non-feeding imagos. In this species, the fat content of males in the initial phase of their imaginal life is 27.3%, whereas that of the females is merely 16.5%. (Expressed in dry weight per cents, 59.70, and 39.60 respectively!) These extremely high fat content data leave no doubt that the muscular work of flight as based on the energy source of fat is rather extensive in the group of insects with non-feeding imagos. A number of other literature data attest that a similar utilization of fats is not infrequent in other insects. WEIS-FOGH [52] is of the opinion that certain insects utilize fat directly during flight. They have shown that locusts store a great amount of fat prior to their migratory flights. The respiratory quotient (RQ) of *Schistocera* indicates that it is this fat which the animals oxidize on the wing. [24]. According to WIGGLESWORTH [56], lepidopterous imagos are unable to utilize carbohydrates during flight, and they have to convert it first into fats. ZEBE [60] makes a similar statement. He claims that if glucose be injected into the lymph of lepidopterous imagos, it will be converted into fat to supply energy for flight.

The last column of Table 10 presents calculation data on the amount of fatty substances found in male gipsy moths of different ages and average weights. The mean weight data necessary for the calculations have been read from the graph plotted on the basis of Table 4. These data show that the fat reserves of the moths do not decrease till they are 24 hours old, indeed, the amount slightly increases. One might contend of course, that this result is coincidental with and derives from the individual fluctuation in the weights and fat contents of the animals used in the experiments. However, GILBERT and SCHNEIDERMANN [18] submit an interesting account to the effect that the non-feeding imaginal males of the *Hyalophora cecropia*-group also convert foreign substances into fat. It was demonstrated by biochemical methods that this transformation takes place in the flying-muscles of the animal. It is quite possible that the same process occurs also in the closely related *Lym-*

antria dispar. This assumption is substantiated by the observation that 33 hours old, still actively flying males contain yet a high amount of fat. The final settlement of the question requires future study.

During the flight of the male gipsy moths, the fatty reserves decrease apart from the initial phase mentioned above (Table 10). The animals gradually utilize their reserve substances. This fact, as well as the marked decrease of the body weight, together with the considerable difference between the fat contents of the two sexes, fundamentally delimit the gipsy moth — and surely all other related species — from the other insects with feeding imagos. These latter animals namely, let they be either holometabolic or not, do not lose regularly (at least as far as we know) neither their fat contents nor their body weights in the imaginal stage, nor is there any conspicuous sexual difference as to fat content. The developments of their weights and fat contents depend much more on the seasons or other external factors of the habitat than on their age. The fat content of *Tenebrio* imagos varies between 4 and 6% both in young and aged specimens [35]. The fat content of *Eurygaster integriceps* and *Leptinotarsa decemlineata* imagos changes according to the season, that is the diapausas connected with them [46]. *Leptinotarsa* contains, in August, only about 8—9 dry weight % fats, about 38—39 in September, at the beginning of the diapausa. There is hardly any difference in this respect between males and females.

Table 10 also shows that the male *Lymantria* imagos under study contain 10.39 living weight % fats (4.1 mg), even at the time of their death. This observation obviously raises the question whether these moths live by nature until their reserve substances suffice, and die only when they have completely exhausted them. There is a possibility that the animals kept under laboratory conditions, however well cared for, had an unnaturally shortened life span incurring the retention of a fragment of their fat contents. FERNALD considered it probable that the males live longer in the field than in the laboratory [37]. In my opinion even if there be some truth in this assumption, the remaining fat content could largely be accounted for by its forming an integral part of the imaginal tissues, hence unconvertible for energy purposes. LAFON [26] is of the same view when he states that *Phormia* specimens utilize but 69% of the fats during their life span of 4—5 days. It would seem therefore that organisms of this type live in fact until the reserve substances cover their energy requirements; one should add, however, that they cannot utilize a certain quota of their energy reserves. BALÁZS, KOVÁTS and BURG [3] discussed the problem of the death of insects with non-feeding imagos. They have investigated *Galleria mellonella*. Of changes observed in the imagos (rate and developments of the decrease of weight, high dry matter losses, increase of relative ash content, rate of decrease of total lipoids, etc.), they inferred that the death of these specimens was due not so much to a general ageing process but to hunger. This suggestion agrees entirely with the above considerations.

Though there remained some fatty substances in the experimental specimens at the time of their death, they were of a rather poor amount, and ultimately, it was more surprising than not that the animals had utilized the major portion of their original fat reserves. If the amount of fat present in the fresh pupae was twice as much as what had been in them before hatching, then only about 22% of the fat reserves of the fresh pupae remained in the male imagos by their death.

Table 11

The lipid content of the copulated female Lymantria dispar imago and its eggs

Designation of age	Number of specimens examined	Lipid content (per cent of live weight)	Lipid content in specimen (and its eggs) of average size (mg)
Imago at hatching	8	4.49	
Idem	10	4.63	
Mean value		4.56	18.4
Imago, 20 hour age	10	5.50	17.9
Imago, at cessation of oviposition	15	2.09	
Idem	6	1.89	
Mean value		1.99	1.6
Egg-cluster 0—48 hour age	15	7.82	
Idem	6	7.26	
Mean value		7.54	14.8
Imago, after oviposition, premortal stage	10	2.26	1.7

The fat content of females, at their hatching from the pupae, is 4.56 live weight %; slightly lower than that of the males of similar age (Table 11). However, by the fact that their weight decreases less than that of the males their relative fat content will later increase also less. This results in a considerable difference, within a few hours, in the relative fat content of the male and the female gipsy moth imagos. I have already mentioned that, according to the available literature, this kind of sexual difference is highly characteristical of non-feeding imagos. Moreover, GILBERT and SCHEIDERMANN [18] believe that the males of lepidopterous species have in general a higher fat content than the females.

The fat content of the females is, despite what was said above, significant in an absolute sense. The animal needs this amount of fat reserve primarily for the production of eggs. This process namely requires a considerable quantity of fatty substances. This observation must obviously be related to the

fact that, in contrast with *Lepidoptera*, the females of other insect orders have in general a higher fat content [18]. According to investigations, the major portion of fats shown in the females of the gipsy moth is transmitted into the eggs. The data of Table 11 prove that a single cluster of eggs contains roughly nine times as much fat-lipoid substances as the female which laid the eggs. It goes without saying that a great amount of energy is needed to meet the exigencies of the embryonal development. This energy is primarily afforded by the 7.54 per cent fat content of the eggs. The importance of the fat content of the insect eggs was confirmed already by TANGL [43, 44]. WIGGLESWORTH [55], referring to the studies of FARKAS and TICHOMIROFF, reports that in the eggs of the silkworm two-thirds of the required energy derives from fat, and that the fat content decreases from 8.1% to 4.4% during embryonal development. The rest of the energy ($^1/_3$ part) is supplied by glycogen. According to SLIFER [38, 39] the eggs of *Melanoplus* initially contain 17—22% dry weight fat, which decreases to half this amount until hatching. HILL [19] stated that glycogen is chiefly responsible in the formation of chitin.

With respect to the high fat content of the eggs, it is especially striking that the females have but a small amount of fatty substances to cover their own energy requirements (Table 11). This proves again that these animals utilize only a meagre amount of energy in meeting the exigencies of their biotic processes.

Water

It is well known that the water content of the larvae is rather high, but rather low in the pupae. However, the male pupae of *Lymantria dispar* contain relatively much water. In the period before hatching I have found 78.11% water in them (Table 12). The pupa of *Hyphantria cunea* contains only 70—74% water [14]. LUDWIG and LANDSMAN [55] found also 74% of water in the pupa of *Popillia japonica*. There is still less water, as reported by SUBLKEV [25], in the pupae of *Corymbites tesselatus* and *Agriotes lineatus*, the former containing 59.98%, the latter 58.7% water. However, this water reserve hardly diminishes in general during metamorphosis, as was satisfactorily shown for *Hyphantria cunea* [14] and *Leucoma calicis* [20]; indeed, the relative water content even exhibited some increase in the former case. The explanation is that oxidation processes necessary for obtaining energy yield a considerable amount of water, largely covering evaporation losses in the pupa. However, there are again exceptional cases. The weight of the pupa of *Musca domestica* decreases by one-third during metamorphosis lasting for 6 days [22], but the major portion of decrease takes place in the first 36 hours. The initial strong decrease in weight is mainly due to the loss of water. JÁNSKY explains this by the fact that the external waxy cover of the pupa preventing evaporation in later periods had not yet developed at that time.

Table 12

The water content of the male Lymantria dispar imago

Designation of age		Number of specimens examined	Water content (per cent)	Water content in specimen of average weight (mg)
Pupa before hatching of imago		10	78.11	181.8
Imago at hatching		10	74.86	111.0
Imago, 4 hour age		10	60.67	
Idem		15	58.77	
Mean value			59.72	53.9
Imago, 24 hour age		10	56.73	
Idem		8	56.33	
Mean value			56.53	
Imago, 36 hour age		12	58.15	
Imago, 51 hour age		10	50.62	
Idem		1	49.59	
Mean value			50.10	
Imago, 72 hour age		10	56.53	
Imago, 78 hour age		3	49.73	
Imago, 52 hour age	dying	1	48.90	
Imago, 70—80 hour age	dying	10	51.10	
Imago, 78 hour age	dying	1	53.84	
Imago, 82 hour age	dying	1	50.46	
Imago, 82 hour age	dying	1	52.43	
Imago, 83 hour age	dying	1	51.45	
Mean value	dying		51.36	20.3

Between the fat and water content of insects, there usually is, as was only to be expected, a connection expressible as an inverse ratio. This has been demonstrated already on larvae. THEODOR [45] mentions that the fat content of the active larva of *Phlebotomus papatasii* is 5% of the dry weight, whereas the water content is 65—70%. During hibernation, these values are modified to 15, and 52—57% respectively. USHATINSKAYA [46, 47] also discusses the water to fat ratio stating that, under the continental climate, insects feed more intensively previously to the diapause. A significant fat reserve is built up, with the concurrent decrease of the water content. The lower water content of the

pupae, as related to that of the larvae, is also connected with the fact that the fat reserves of the animals generally increase during pupation. The larvae of *Hyphantria cunea* contain 1.3 — 2.0% fats and lipoids, and about 84—86% water. When, however, their fat content increases to 4.2%, the water level decreases to 81.4% [4, 15]. This conspicuous process of building up the stores of energy reserves continues until the beginning of the pupal stage. The 8.87% fat content of the young *Hyphantria* pupae coexists with an approximate 70% water content.

The water and fat content relationship is particularly striking in pupae which show sexual differences as to the amount of fat reserves. Such relationship is known, among others, in the case of *Leucoma salicis*, studied by JANDA and MAREK [20].

Knowing the inverse ratio of water and fat contents, one should only expect that the water content of the male *Lymantria dispar* imagos will decrease in the first hour of their lives, concurrently with that increase of their per cent fat content. The data of Table 12 indeed display that in that phase, the same substantial decrease in water content takes place in their organisms as there was a notable increase in their relative fat content. In this period, namely, the animal gives off all its dispensable water reserves, which again promotes the reduction of their body weight to its smallest possible amount. There is hardly more than 56.5% water content in 24 hour old imagos. In later phases, it is remarkable that the relative water content diminishes only slightly, and not all in the second half of the period of flight. This fact, combined with the observation that the imago starts on its flight with a rather meagre amount of water, imply that this species utilizes water derived from the various oxidizing processes taking place in its organisms. The reserve substances meeting the requirements of the flight are therefore utilized, as it were, twice by the animal: first by obtaining energy during the oxidizing processes, secondly by satisfying its water requirements by water emerging as the final product of the same processes.

I have grouped in Tables 13 and 14 the water content of uncopulated and copulated females and their eggs. As is to be seen the water content of females just hatched from the pupae is only 75.42%, scarcely half per cent higher than that of the males of the corresponding age. Subsequently, however, there evolves a considerable difference between the two sexes. The water content of the females does not decrease by far in the same degree as that of the males. This phenomenon again shows, on the one hand, that the females have no need — since they do not fly — to free themselves of all dispensable materials for the sake of lessening their weight as do the males, and, on the other, that the interrelationship of the fat and water content holds as discussed above.

The water content of uncopulated females (which lay only a part of their eggs) is hardly lower even at their death than in the first 24 hours of their

life (Table 13). The slight loss of weight and moderate loss of water are almost proportionate in this phase of their life.

With regard to copulated and oviposited females, there are two observations worthy of note. First, the water content of the eggs is lower than that of their own bodies. This also follows from the higher fat content of the eggs and the lower one of the imagos. Secondly, one should pay attention due to the

Table 13

The water content of the uncopulated female Lymantria dispar imago

Designation of age	Number of specimens examined	Water content (per cent)	Water content in specimen of average size (mg)
Imago at hatching	8	76.50	
Idem	5	74.55	
Idem	10	75.22	
Mean value		75.42	303.7
Imago, 24 hour age	10	73.43	
Idem	10	73.58	
Mean value		73.50	239.2
Imago			
106 hour age, dying	2	73.18	
120 hour age, dying	1	72.13	
98 hour age, dying	1	73.45	
100 hour age, dying	1	71.89	
Mean value		72.66	199.8

fact that the water content of the females decreases rapidly and conspicuously after oviposition until their death. Indeed, this process begins already during oviposition. Though the oviposition of the eggs containing a relatively smal amount of water should increase the relatively low water content of the females this growth fails to occur. During the last 24 hours of its life the animal loses almost 4% of its water content, thus the loss of water approaches the rate of the decrease of the whole live weight occurring during this period (Table 9). At the same time, however, as testified by Table 11, the amount of fat still extant in the animal does not decrease. This would imply that the metabolism, slow as it already is, of the female decreases to an extraordinary degree after oviposition and the animal nearly dries up at the time of its death.

The difference arising from the divergent development of the water contents of uncopulated and copulated females calls attention to the fact

that these latter perish in other physiological conditions than the former ones. The water content conditions of the gipsy moth males and uncopulated females substantiate the hypothesis, mentioned above, of BALÁZS, KOVÁTS and BURG [3] on the inanitional atrophy of insects with non-feeding imagos. However, the decrease of the water content raises the idea whether the copulated and oviposited females have not died due to other causes, possibly owing to natural ageing processes. Such difference among the causes evoking death would theoretically be understandable, since the process of oviposition is unrepeatable for

Table 14

The water content of the copulated female Lymantria dispar imago and its eggs

Designation of age	Number of specimens examined	Water content (per cent)	Water content in specimen (and its eggs) of average size (mg)
Imago, at cessation of oviposition	2	73.67	
Idem	2	72.00	
Idem	5	74.92	
Idem	8	74.06	
Mean value		73.66	59.6
Egg-cluster 0-48 hour age	2	66.76	
Idem	2	66.86	
Idem	5	68.05	
Idem	8	67.35	
Mean value		67.25	131.7
Imago after oviposition, premortal stage	10	69.76	
Idem	5	69.98	
Mean value		69.87	54.0

the female which, after this act is over, has irrevocably finished with its reproductive function, followed in insects generally by natural death. (For all that, this form of death would not preclude that the animal simply utilizes its reserve substances until that time.) In the case of uncopulated females, which had therefore failed to perform their duties in the propagation of the species, there can be no question of a similar end to the imaginal life. They will presumably live and wait for fertilization until their reserve substances hold out. The males resemble these latter inasmuch as being animals capable of repeated copulation they do not attain the state of having completely dispensed with their functions in propagating the species. All these considerations are supported by the observation that the water content of insect species with feeding imagos decreases in the last phase of their life, similarly to the oviposited gipsy moth. And their

death is doubtless due to ageing and not to starvation. For instance, according to the findings of Cook and Scott [8], the water content of *Termopsis* is 74—80%, but this amount decreases to below 68% soon before its death. One should not forget, however that one cannot safely regard the decrease of water as symptomatic of ageing. According to Balázs, Kováts and Burg [3], the change of the nitrogen, ash and fat contents is a better indicator in this respect. Nor should one overlook the fact that the life span of uncopulated females is not at all longer but rather shorter than that of the copulated ones. A satisfactory solution of the problem requires further study.

The production biological evaluation of the research data

I have already referred in the introduction to the special position of holometabolic insects with non-feeding imagos within the several communities. Their state has been elucidated from several sides by the investigations discussed above. In how far is, therefore, the position of the animals in question — among them that of *Lymantria dispar* — special as mirrored in the present research? In answering this question, I think that we could sort out three inferences derivable from our study.

First of all, these moths are characterized by a high grade of adaptation and specialization. Their whole organic structure has profoundly specialized itself to subserve the propagation of the species. They do nothing, as it were, which is not connected with this task.

Secondly, and this is closely related with the first point, these animals are characterized by an extreme economy of materials and energy.

Thirdly, it is especially striking in how far and to what rates they consume, subsequently to the cessation of feeding, their own organism at the end of the larval stage and during the pupal and imaginal stages. Should the problem be approached either as concerns the developments of body weight or from an energetical point of view, it is equally astounding how little there remains of the animals after reproduction, at the time of their death.

The above considerations are naturally connected with the fact that the animals cease feeding early and finally, and then depend exclusively on their reserve substances, including water. The observer sees, on the one side of these interrelated phenomena, the successful fulfillment of the tasks of reproduction, and on the other the almost unbelievable decrease of the weight of the males during flight, their freedom from carrying any excessive burdens, their combustion of mainly fats which contain a relatively high amount of energy despite the small quantities, their utilization of water derived during oxidation, their saving of energies by avoiding all unnecessary movements, as well as the devoting by the females — for all their modest metabolism — of all their forces to the development of eggs and to oviposition — in short, the utilization by the animals of all available energies and materials.

The high amount of losses in energy and matter, observable from the time of the cessation of feeding until death, deserves especial attention from the point of view of production biology. Living beings — and here I have in mind heterotrophic, that is, transferent, organisms [17] — increase, at least in some phase of their life, the energy and material reserves of their own bodies by a part of the consumed food, to wit they augment the materials and energies stored (as parts or reserves of the organism) — in short, they "store actively." The metabolism of a number of organisms changes in a given phase of their life so that the amount of energy and materials, consumed as food on the one hand and freed and oxidized as energy on the other, will in essentials strike a balance. These animals do not, therefore, increase the energy and material reserves of their bodies but merely retain the extant amount, hence they "store passively." Also those organisms are "passively storing" which lose more materials and energies than what they take up in the food. These reduce their existing material and energy reserves, retaining only part of it. Into this latter category can the individuals of *Lymantria dispar* and similar animals be relegated, subsequent to the cessation of their food uptake. However, the rate of the loss of the stored materials and energy attain such extremes that, by this very fact, they are wholly distinct from all other organisms. The weight of the male moth at its death is no more than 6.6% of its maximum weight in the larval stage, and if one starts from the assumption that these animals utilize about half of their fatty substances in the pupal stage, approximately 78% of this amount will be lost at the end of their life. In judging the females one might say that the major portion of their available reserves is transmitted into the eggs as living materials — but the production of offsprings is a characteristic of the members of all groups of living beings. Thus the weight and energy ratios of the females ought to be considered in this case regardless of the eggs. And accordingly, they exhibit a loss of weight greater even than that of the males, while their fat reserves become quite insignificant at the end of their life. These animals therefore decompose their own organism to such a rate that there remains, after their death, but little of them for utilization by other organisms feeding on dead organic materials. They extract, as it were, the materials of their body from the community. This is the reason why their position is singular from the point of view of production biology, and this is the justification for designating them as a distinct group of living beings: perhaps as self-decomposing organisms.

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UNTERSUCHUNG DER GEWICHTSVERÄNDERUNGEN, DES FETT- UND DES WASSERGEHALTES BEI *LYMANTRIA DISPAR L.* (MIT BESONDERER BERÜCKSICHTIGUNG DER CHEMISCHEN UND ENERGETISCHEN VERÄNDERUNGEN WÄHREND DER METAMORPHOSE SOWIE IM LAUFE DES LEBENS DER IMAGINES VON INSEKTEN)

In der vorliegenden Arbeit berichtet der Verfasser über die Ergebnisse seiner Untersuchungen, die er an den Puppen und den Imagines von *Lymantria dispar* vornahm. Die Resultate der Lebensdauer-, Fettgehalts- und Wassergehaltsuntersuchungen sprechen dafür, daß die *Lymantria dispar* Insekten eigenartig spezialisierte Organismen sind. Ihre Spezialisierung steht damit in Zusammenhang, daß die Imagines dieser Insekten keine Nahrung aufnehmen und deshalb ihren Bedarf an Nährstoffen und Energie aus den im Larvenstadium angehäuften Reserven decken. Sie verwenden diesen Vorrat weitgehend zur Sicherung der Fortpflanzung. Da sie keine Nahrung aufnehmen, bauen sie ihre eigenen Körpersubstanzen ab, und zwar bis zum Ende ihres Lebens so hochgradig, daß es vom produktionsbiologischen Gesichtspunkt aus berechtigt erscheint, diese Tiere mit einem besonderen Namen als Selbstverdauer zu bezeichnen. (Zweifellos kommt dieser Name auch den anderen holometabolen Insekten zu, die im Imagostadium keine Nahrung aufnehmen.) Dies ist sowohl für die männlichen als auch für die weiblichen Imagines kennzeichnend, in bezug auf die Verwertung der Nährstoffreserven läßt sich jedoch zwischen den Individuen der beiden Geschlechter, die auch in morphologischer Beziehung verschieden sind, ein Unterschied nachweisen. Nach Beendigung der Nahrungsaufnahme der Raupen ist der Gewichtsverlust der Männchen dermaßen groß, daß das Gewicht des im Eingehen begriffenen Schmetterlings nicht mehr als 6,6% des Maximalgewichts der Raupe ausmacht. Der Gewichtsverlust wird auch noch dadurch erhöht, daß die Imagines beim Ausschlüpfen aus der Puppe von jedem überflüssigen Stoff befreit werden, wodurch ihr Gewicht auf das möglichst geringste reduziert wird, und sie die Muskelarbeit des Fluges durch verhältnismäßig geringen Energieverbrauch bewältigen können. Der vorhandene Energievorrat wird fast ausschließlich zum Fliegen verbraucht, das im Dienste der Paarsuche steht. — Der Fettgehalt des männlichen Schmetterlings ist sehr hoch und beträgt im Alter von 33 Stunden 18,16% des Lebendgewichts. Während des Fluges wird dieser Fettvorrat allmählich verbraucht, obwohl ein geringer Teil noch beim Eingehen des Schmetterlings vorhanden ist. — Der Wassergehalt vermindert sich während des Ausschlüpfens aus der Puppe und in den ersten Stunden des Schmetterlingslebens erheblich. Im Körper des 24 Stunden alten männlichen Schmetterlings läßt sich bereits kaum 56,5% Wasser feststellen. Später nimmt allerdings der relative Wassergehalt nur wenig und in der zweiten Hälfte der Flugzeit überhaupt nicht mehr ab. Dies läßt sich damit erklären, daß das Tier das während der Oxydation des Fettes entstehende Wasser verbraucht. Während die Männchen den Vorrat an Nährstoff-Energie für den Flug verbrauchen, verwenden ihn die Weibchen weitgehend zur Erzeugung der Eier. Die ruhigen, sich kaum fortbewegenden Weibchen verbrauchen für die Sicherung ihres eigenen Stoffwechsels weit weniger Nährstoffreserven als die Männchen. Legen sie keine Eier, so nimmt ihr Gewicht während ihres Schmetterlingslebens um 31,7% ab. Auch ihr Fettvorrat, der übrigens verhältnismäßig bedeutend geringer ist als die Fettreserve der Männchen, ändert sich wenig. Nach der Eiablage zeigen aber die Weibchen ein ganz anderes Bild. Das Gewicht solcher Tiere beträgt vor ihrem Eingehen etwa 19,2% ihres Gewichts nach der Entpuppung. Das Gewicht der gelegten Eier beträgt hingegen 48,6% des Gewichts der jungen Schmetterlinge. Ein erheblicher Teil der Fettstoffe (etwa 80% des ursprünglichen Fettvorrats) gelangt ebenfalls in die Eier. Nach der Eiablage hat dagegen das Weibchen einen höheren Wassergehalt als seine Eier, ein Beweis mehr, daß das Tier die Substanzen mit höherem Energiegehalt an die Eier weitergibt.

ИССЛЕДОВАНИЕ ИЗМЕНЕНИЙ ВЕСА, СОДЕРЖАНИЯ ЖИРОВ И ВОДЫ У НЕПАРНОГО ШЕЛКОПРЯДА (*LYMANTRIA DISPAR L.*) (С ОСОБЫМ УЧЕТОМ ХИМИЧЕСКИ-ЭНЕРГЕТИЧЕСКИХ ИЗМЕНЕНИЙ, ПРОИСХОДЯЩИХ ВО ВРЕМЯ МЕТАМОРФОЗОВ НАСЕКОМЫХ И В ТЕЧЕНИЕ ЖИЗНИ ИМАГО)

В настоящей статье сообщаются результаты исследований, проводившихся на куколках и имаго непарного шелкопряда (*Lymantria dispar L.*) Исследования продолжительности жизни, изменений веса, содержания жира и воды указывают на то, что непарный шелкопряд (*Lymantria dispar L.*) является своеобразно дифференцированным организмом. Специализация состоит в том, что в стадии имаго эти насекомые не принимают пищу и, следовательно, они используют для покрытия своих потребностей в питательном веществе и энергии резервы, накопившиеся в личиночной стадии. Эти резервы они в значительной мере используют для обеспечения размножения. Ввиду того, что они не принимают пищу, непарные шелкопряды до конца своей жизни в такой большой мере расщепляют вещества своего собственного тела, что вполне обоснованно назвать этих животных особым названием — самопереварителями. (Несомненно, что остальные голометаболические насекомые, не принимающие пищу в стадии имаго, также следует назвать этим названием.) Это относится, равным образом как к самкам, так и к самцам, однако, в отношении использования резервных питательных веществ между особями двух полов наблюдается значительная разница, точно так же, как и в отношении морфологии. После окончания питания гусениц, у самцов наблюдается такая значительная потеря веса, что вес погибающей бабочки не превышает 6,6% максимального веса гусеницы. Потеря веса повышается еще и тем, что имаго при вылуплении из куколки освобождаются от всех излишних веществ, причем их вес снижается до минимума, благодаря чему они при полете могут обеспечить работу мышц за счет относительно меньшей энергии. Имеющиеся резервы энергии они израсходуют почти исключительно для полета в целях нахождения самки. — В теле самца содержание жира весьма большое, в 33-часовом возрасте оно составляет 18,76% живого веса. Во время полета это депо жира постепенно израсходуется, хотя незначительная часть его сохраняется до гибели бабочки. — Содержание воды во время вылупления из куколки и в течение первых часов жизни сильно убывает. В теле 24-часовой мужской бабочки содержание воды составляет лишь 56,5%. Однако в дальнейшем оно мало изменяется, а во второй половине времени полета относительное содержание воды уже вовсе не снижается. Этот факт объясняется тем, что животное использует воду, возникающую в процессе окисления жира. — В то время как самцы используют резервы питательных веществ-энергии главным образом для полета, то самки израсходуют эти депо прежде всего для продукции яиц. Самки ведут спокойный образ жизни, они почти не передвигаются и, следовательно, для обеспечения своего собственного обмена веществ они используют гораздо меньше энергии, чем самцы. Если они не кладут яиц, то их вес уменьшается в течение жизни бабочек лишь на 31,7%. Их содержание жира — которое, впрочем, гораздо меньше содержания жира в теле самцов — также мало снижается. Однако яйценоская самка показывает совершенно иную картину. Вес яйценоской бабочки составляет при ее гибели в среднем 19,29 % веса, измеренной после ее вылупления из куколки. Вес яиц же равен 48,6% веса молодой бабочки. Значительная часть жировых веществ животного (около 80% первоначального резерва жира) также попадает в яйца. После яйцекладки содержание воды в теле самки больше, чем в яйцах. Это является лишним доказательством того, что насекомое передает яйцам вещества с более высоким содержанием энергии.

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Acta Biol. Hung., **15** (2), 171—179 (1964)

THE ADAPTATION TO SALTWATER CONDITIONS OF THE ADRENAL STRUCTURE IN VARIOUS BIRD SPECIES

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(Received February 10, 1964)

Synopsis

In the course of the present study, the adrenal of the kentish plover *(Charadrius alexandrinus alexandrinus* L.), the dunlin *(Calidris alpina* L.), the common sandpiper *(Actitis hypoleucos* L.), the lapwing *(Vanellus vanellus* L.), the tree-sparrow *(Passer montanus montanus* L.) and the pigeon *(Columba livia domestica* L.) had been examined histologically and histochemically. In the adrenal of *Actitis hypo eucos*, living along freshwater brooks and rivers, there is no difference as to the lipoids between the peripheral and the deeper-lying interrenal cells. The peripheral cells are slightly larger, their nuclear volume greater than that of the deeper situated cells. In the case of the common sandpiper, one could therefore assume a rate of aldosterone production similar to those birds (lapwing, tree-sparrow, pigeon) which consume usually "freshwater" only. The sudanophily in the adrenal of *Charadrius alexandrinus alexandrinus* inhabiting sodic areas and *Calidris alpina* of the seashore shows a certain degree of zonation. The nuclear volume of the peripheral interrenal cells is substantially smaller than that of the deeper-lying interrenal cells. This finding points to aldosterone production on a smaller scale, which can be explained by the overstressing of the organism by sodium salts. The decreased aldosterone secretion of the bird species consuming saltwater on sodic areas and seashore is to be interpreted as the biological adaptation of the adrenal

Introduction

In the adrenal of birds the cortical and medullary substances do not separate. There is only one exception, on the basis of investigations up to the present day, namely the pelican *(Pelecanus occidentalis* L.) in whose adrenal the cortical and the medullary substances are up to a certain degree distinct from each other. In this species, the interrenal cells constituting the cortical substance form three zones, similarly to the mammals [3]. It was established later, also by KNOUFF and HARTMANN, that the peripheral zone is more easily activated than the cells of the deeper-lying zones [1].

SINHA and GHOSH [5] found that the peripheral and the deeper lying interrenal cells of the pigeon's adrenal are histochemically different.

ROOS's [4] investigations on the pigeon *(Columba livia domestica* L.), the mallard *(Anas platyrhyncha* L.), the domestic fowl *(Gallus domesticus* L.) and the hering gull *(Larus occidentalis* L.) had shown that their adrenal secretes both aldosterone and glucocorticoids.

Accordingly, the interrenal cells of the avian adrenal, considered earlier as a homogeneous organ, are at least functionally different from each other.

This was demonstrated also by KONDICS's investigations [2] showing that the introduction of salt caused the atrophy of the peripheral part and the hypertrophy of the deeper-lying interrenal cells in the adrenal of the pigeon. Hence it was inferred that the peripheral interrenal cells secrete aldosterone, whereas the deeper-lying ones produce glucocorticoids.

A logical outcome of the above literature data is the question whether an extreme adaptation to special ecological conditions would incur changes in the functioning of the interrenal cells of the adrenal regulating the salt-metabolism of the organism.

To answer the question, it seemed proper to examine histochemically and histologically the adrenal of aquatic bird species of "freshwater" and "saltwater" (sodic) habitats.

Material and method

Five specimens each (males and females) of the species to be investigated had been collected during August and September. These were: the kentish plover *(Charadrius alexandrinus alexandrinus* L.), the dunlin *(Calidris alpina* L.), the common sandpiper *(Actitis hypoleucos* L.), the lapwing *(Vanellus vanellus L.)*, the tree-sparrow *(Passer montanus montanus* L.) and the pigeon *(Columba livia domestica* L.). Materials referring to this latter species had been obtained by the courtesy of L. KONDICS.

The adrenal of the one side was fixed in 5% formalin, that of the other side in Haidenhain's "Susa" solution. Of the material fixed in formalin 15μ sections were made with freezing microtome, and the lipoids were shown by Sudanrot-7B. Of the other adrenal, fixed in "Susa", 5 μ sections were made after double Péterfi-embedding; the sections were then stained with haematoxyline-eosine. Of the peripheral and deeper-lying bundles of the interrenal cells, one hundred nuclei each were measured by a 3000× projecting magnification, their volume calculated by the Fischer—Inke nomogramm, based on the formula $V = \Pi/6\ AP^2$, and the values received plotted on a coordinate system.

Results

The "control" species was the common sandpiper *(Actitis hypoleucos* L.), in which, as concerns sudanophily, there was no observable difference (Fig. 1) between the peripheral and the deeper-lying interrenal cells. The histological pattern is characterized by relatively few adrenal cells embedded among the interrenal cells constituting a great number of bundles. As to size, the peripheral and the deeper-lying layers of the interrenal cells cannot (or hardly) be distinguished from one another (Fig. 2). It is only on the basis of the nuclear volumes that a minimal difference can be shown between the two zones (Fig. 7*A*). The nuclei of the peripheral interrenal cells are namely slightly larger than those of the interrenal cells lying below them. In the staining of the cytoplasm, the peripheral and the deeper-lying interrenal cells agree.

In order to make our findings more general three additional bird species had been investigated, which consume only "freshwater", that is, ingest "saltwater" only occasionally.

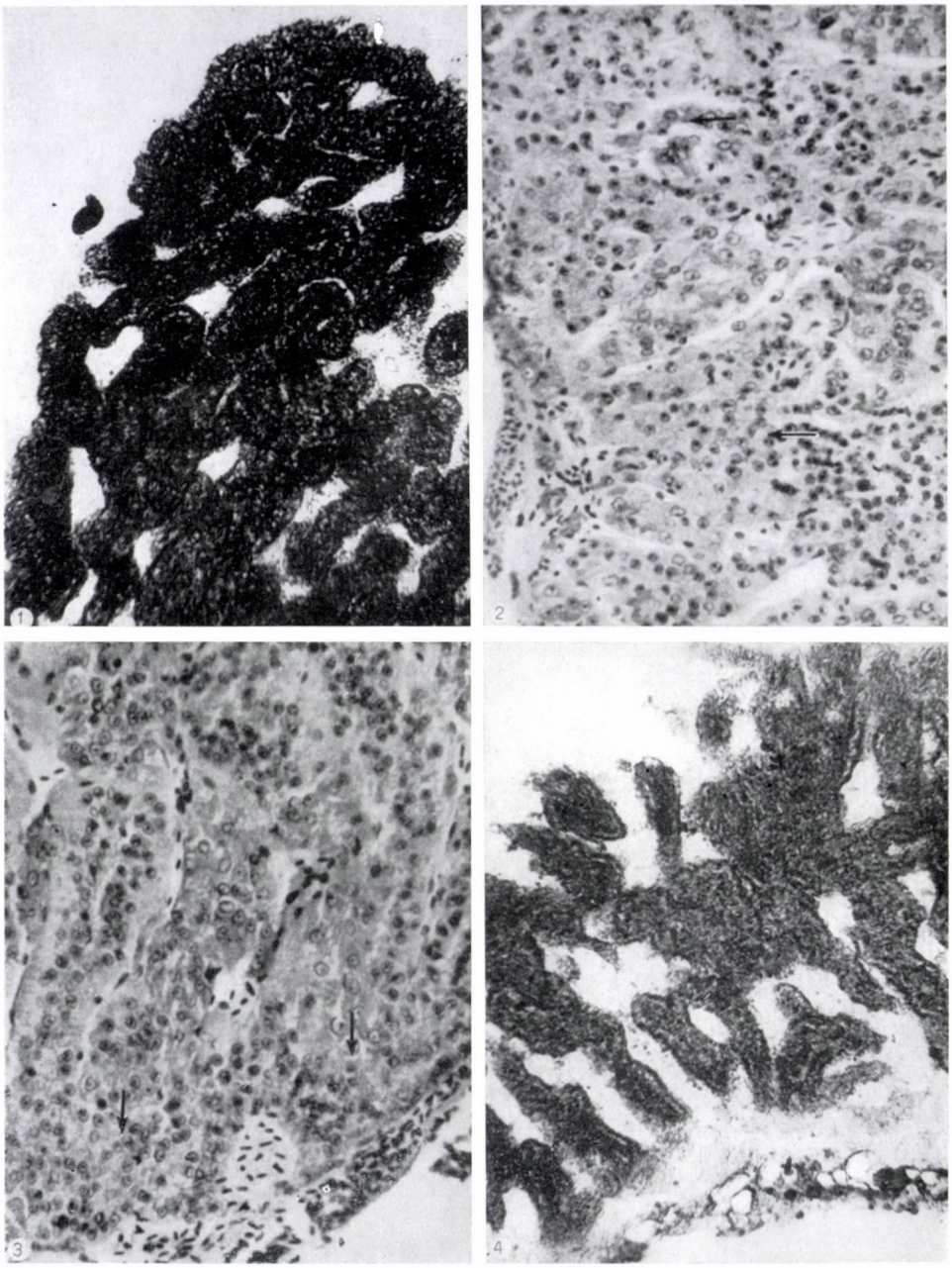


Fig. 1. Actitis hypoleucos L. Fixation in 5% formalin; 15μ freezing section; Sudanrot 7B $\times$ 150

Fig. 2. Actitis hypoleucos L. Fixation in Heidenhain's "Susa"; 5μ paraffine section; haematoxyline-eosine; $\times$530, → border of zones

Fig. 3. Charadrius alexandrinus L. Fixation in 5% formalin; 15μ freezing section; Sudanrot 7B; $\times$150

Fig. 4. Charadrius alexandrius L. Fixation in Heidenhain's "Susa"; 5μ paraffine section; haematoxyline-eosine, $\times$430, → border of zones

The sudanophily of the interrenal cells of the lapwing *(Vanellus vanellus* L.) is characterized by the fact that the peripheral part is poorer in lipoids than the cells of the deeper-lying zone. A striking feature of the histological pattern is the greater size of the peripheral interrenal cells as compared to that of the deeper-lying zones. Their nuclei are more spherical, their cytoplasm staining paler with eosine. The deeper-lying interrenal cells are arranged in thick bundles; their nuclei are smaller and also denser, since the cytoplasmic mass of these cells is smaller than that of the peripheral cells. On the other hand, the acidophily of the cytoplasm is more pronounced in this zone.

In the case of the tree-sparrow *(Passer montanus* L.), the lipoid content of the peripheral and of the deeper-lying interrenal cells is almost identical. The histological picture is characterized by the peripheral interrenal cells being larger than those below them. Their cytoplasm stains less with eosine, the nuclei are larger and more spherical. The deeper-lying interrenal cells, usually arranged into longitudinal bundles, are smaller, the acidophily of their cytoplasm stronger, their nuclei smaller.

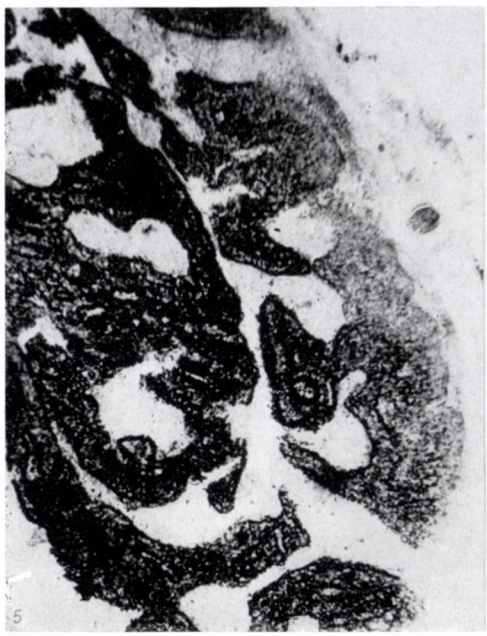
The sudanophily of the peripheral and deeper-lying interrenal cells of the pigeon *(Columba livia domestica* L.) differs hardly or not at all. As concerns the histological pattern, the peripheral and the deeper-lying interrenal cells are of almost equal size, that is, the peripheral cells might be slightly larger. The peripheral interrenal cells stain paler with eosine than those of the profounder zone.

Of the birds ingesting an extreme amount of salt with their food and water, the kentish plover and the dunlin, inhabiting the sodic inland areas and the seashore were examined.

In the case of the kentish plover *(Charadrius alexandrinus* L.), some parts of the peripheral zone are poorer in lipoids than the deeper-lying zone (Fig. 3). It is conspicuous in the histological picture that the peripheral interrenal cells are substantially smaller than those of the deeper-lying zone. They frequently form spherical cell-nests (zona glomerulosa?), which are easily distinguishable from the deeper-lying columellar fascicles of cells (Fig. 4). Their cytoplasm stains stronger with eosine than the deeper-lying interrenal cells, and their nuclei are also smaller, usually elipsoid. The deeper-lying interrenal cells seem to be hypertrophysed as related to the peripheral zone; their cytoplasm is less acidophilous, the nuclei considerably larger, more spherical (Fig. 7*B*).

The lipoid content of the peripheral interrenal cells of the dunlin *(Calidris alpina* L.) is smaller than that of the deeper-lying interrenal cells (Fig. 5). The peripheral and the deeper-lying layer of the interrenal cells are well recognizable and distinguishable on the histological picture (Fig. 6). The peripheral cells are significantly smaller than those lying below them; their cytoplasm stains more vividly with eosine, the nuclei are elipsoid, considerably smaller

than those of the deeper-lying interrenal cells. These latter are usually arranged into longitudinal columns, and also larger than the peripheral ones situated above them. Their larger cytoplasmic mass stains paler with eosine; the nuclei are large, usually spherical (Fig. 7*C*).



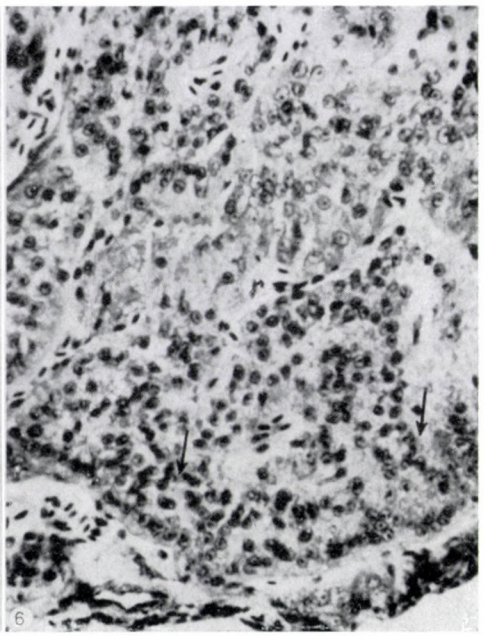


Fig. 5. Calidris alpina L. Fixation in 5% formalin; 15μ freezing section; Sudanrot 7B; $\times$150
Fig. 6. Calidris alpina L. Fixation in Heidenhain's "Susa"; 5μ paraffine section; haematoxyline-eosine; $\times$430, → border of zones

Discussion

In the course of this study, the interrenal cells of the adrenals of three ecotypes have been compared. The two main criteria in the selection of the bird species have been that (1) they be closely related and of similar habits, while, at the same time, (2) the salt content of the water present in their habitats differ considerably from each other (emphasis being mainly laid on sodium salts).

Of the investigated species, *Actitis hypoleucos* belongs to the first ecotype. Its nesting sites are along the upper and middle reaches of brooks and rivers; its migration follows also mainly the course of rivers. Thus the bird obtains its food along the banks of "freshwater". The amount of the ingested sodium salts is meagre, and it corresponds to that of any other animal dependent on "freshwater".

The interrenal cells of the adrenal of this species fail to show any striking functional or morphological zonation. With reference to the nuclear volume, the peripherally situated interrenal cells are slightly larger, i.e., they seem to be more intensely functioning than the deeper-lying interrenal cells. The inter-

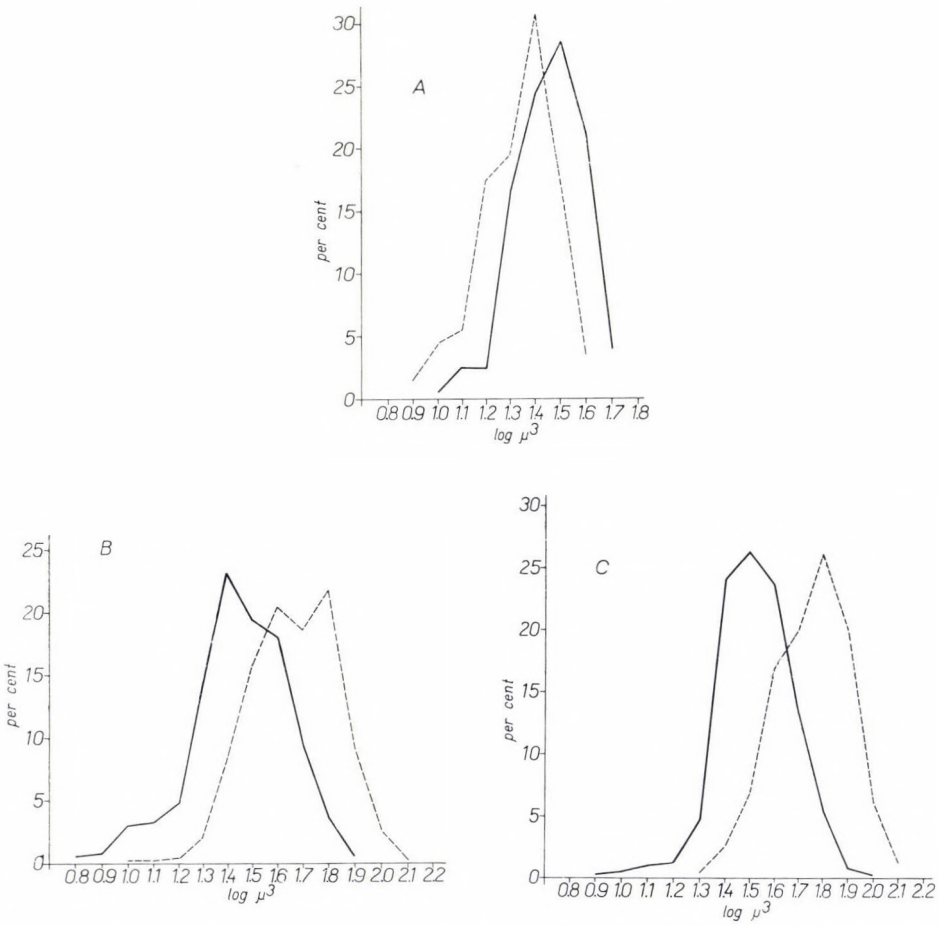


Fig. 7. Nuclear volumes of peripheral and deeper-lying interrenal cells. *A — Actitis hypoleucos* *B — Charadrius alexandrinus* L. *C — Calidris alpina* L. —— peripheral cells; ------ deeper lying cells

renal cells of the adrenal of the lapwing, the tree-sparrow and the pigeon display a similar picture. In all of these birds, the peripheral zone can be characterized by cells larger than or of nearly equal size with the cells of the deeper-lying zone. In the common sandpiper, the lipoid content of the peripheral and the deeper-lying interrenal cells is identical. The corresponding values are the same or almost the same with the tree-sparrow and the pigeon.

It seems therefore, in the case of the common sandpiper as well as in other bird species consuming "freshwater", that the peripheral interrenal cells of the adrenal function more intensely than the deeper-lying interrenal cells. This points to active aldosterone production, in perfect harmony with the specific habit, and effecting an increased retention of the relatively small amount of sodium introduced into the organism of the bird.

In the lapwing, the sudanophily of the peripheral part is less as compared with the deeper-lying cells, even though the cells of this zone show a histological pattern pointing to a rather intense function, the same as with the "freshwater" species mentioned above. The phenomenon might be explained by the fact that the birds studied had been collected in sodic areas of the Hungarian Plain. This bird species, occurring in "freshwater" and "saltwater" habitats alike, reacts, in my opinion, to the acute change in habitat (incurring also a change in the amount of sodium entering the organism) by an alteration in the functioning of the peripheral interrenal cells which manifests itself here in the decrease of lipoids. The assumption is substantiated by KONDICS's [2] investigations which showed that strong lipoid depletion takes place in the peripheral interrenal cells of pigeons kept on a diet of high NaCl content.

The second ecotype would comprise the birds characteristical of the sodic habitats. As a typical representative of this habitat, the kentish plover *(Charadrius alexandrinus* L.) was examined. Its nesting and feeding areas lie on extremely sodic territories, and the bird frequents these sites also during migration. It consumes water excessively rich in sodium salts.

The interrenal cells of the adrenal show a very characteristical functional zonation in this species. The peripheral cells are considerably smaller than the deeper-lying interrenal cells. The divergent character of their nuclear volume is very striking. On the other hand, no definite zonation as concerns the lipoid content, could be shown in every examined specimen. During feeding, the kentish plover ingests a great amount of sodium salts which necessitates an increased excretion of the superfluous salt content, — hence the level of aldosterone production is accordingly low. The decreased function is reflected by the fact that the peripheral zone (secerning aldosterone) is atrophied as related to the deeper-lying zone.

The third ecotype represents the so-called "coastal birds" of the habitats of the seashore. Among others, also the dunlin *(Calidris alpina* L.) belongs here. Its nesting area is the arctic seashore, and the bird usually follows shoreline during migration, but will occasionally traverse also the mainland. On such instances, the bird settles for shorter or longer periods preferably on the sodic fields. Both its food and the water consumed during feeding contain a high amount of sodium salts. Since the dunlins examined had been collected during migration on our sodic fields, one should not exclude the possibility that they to a certain degree had already adapted themselves to the new

environment and thus it were highly desirable to examine the functional zonation of their adrenal also in their nesting habitat. Until these investigations are made, it were best to consider the birds of the seacoast as a subtype of the sodic ecotype.

The interrenal cells of the adrenal of the dunlin display a functional zonation entirely identical with that of the kentish plover. The peripheral cells are atrophied and their lipoid content is smaller than of those lying below them. The difference in the nuclear volume of the two zones is rather conspicuous, pointing to the low intensity of aldosterone production in this bird species, to be accounted for by the necessity of eliminating the high amount of sodium introduced into the organism. The organism of birds belonging to the coastal and sodic ecotypes is constantly overstressed by the ingested sodium. Accordingly the aldosterone production in these birds shows a decreased intensity, in contrast with those species which consume "freshwater". The phenomenon must surely be interpreted as a special adaptation of the living organism. The fact that the adaptation of the adrenal in both the sodic and coastal ecotypes is of the same character allows the assumption, in the case of these birds, of a certain physiological correspondence which would explain the frequenting of these very sodic areas by the coastal birds during their migration.

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DIE ADAPTATION DER NEBENNIERENSTRUKTUR VERSCHIEDENER VOGELARTEN AN DIE SALZWASSER-UMGEBUNG

Die Nebennieren folgender Vögel wurden histologisch und histochemisch untersucht: Seeregenpfeifer *(Charadrius alexandrinus alexandrinus* L.), Alpenstrandläufer *(Calidris alpina* L.), Wasserläufer *(Actitis hypoleucosa* L.), Kiebitz *(Vanellus vanellus* L.), Feldsperling *(Passer montanus montanus* L.) und Haustaube *(Columba livia domestica* L.). In den Nebennieren der Wasserläufer, die Bächen und Flüssen entlang siedeln, läßt sich in bezug auf den Lipoidgehalt zwischen den peripheren und tiefer liegenden interrenalen Zellen kein Unterschied feststellen. Die peripherischen Zellen sind aber etwas größer, ihr Kernvolumen ist höher als das der tiefer liegenden Zellen. Beim Wasserläufer können wir also eine intensive Aldosteronsynthese annehmen, ähnlich wie bei anderen Vögeln, die weitgehend nur »Süßwasser« trinken (Kiebitz, Feldspatz, Haustaube). In den Nebennieren der sodahaltigen Seen entlang lebenden Seeregenpfeifer und der an Meeresküsten siedelnden Alpenstrandläufer zeigt die Sudanophilie eine gewisse Zonation. Das Kernvolumen der peripherischen interrenalen Zellen ist wesentlich kleiner, als das der tiefer liegenden interrenalen Zellen. Dies weist auf eine geringe Aldosteron-

synthese hin, was mit der übermäßigen Belastung des Organismus durch Na-Salze zusammenhängt. Die herabgesetzte Aldosteronsekretion bei »Salzwasser« trinkenden Vogelarten, die der Meeresküste entlang und an sodahaltigen Seen leben, kann als biologische Adaptation der Nebenniere gedeutet werden.

ПРИСПОСОБЛЕНИЕ СТРУКТУРЫ НАДПОЧЕЧНИКОВ РАЗЛИЧНЫХ ВИДОВ ПТИЦ К СРЕДЕ С ЗАСОЛЕННОЙ ВОДОЙ

Автор исследовал гистологическими и гистохимическими методами надпочечники морской зуйки (*Charadrius alexandrinus alexandrinus* L.), чернозобика (*Calidris alpina* L.), перевозчика (*Actitis hypoleucos* L.), чибиса (*Vanellus vanellus* L.), полевого воробья (*Passer montanus montanus* L.) и голубя (*Columba livia domestica*). В надпочечниках перевозчиков, живущих вдоль ручейков и рек, в отношении содержания липоидов не отмечается различия между периферическими и более глубоко расположенными интерренальными клетками. Периферические клетки несколько больше, объем ядер больше, чем в более глубоко залегающих клетках. Следовательно, у перевозчиков можно предполагать — подобно как и у других птиц, употребляющих в большинстве случаев исключительно только «пресную воду» (чибис, полевой воробей, голубь), — интенсивную выработку альдостерона. В надпочечниках морской зуйки и приморского чернозобика, живущих в среде с засоленной водой, суданофилия показывает зональность. Объем ядер периферических интерренальных клеток значительно меньше, чем у более глубоко расположенных интерренальных клеток. Этот факт указывает на незначительную выработку альдостерона, что объясняется чрезмерной нагрузкой организма солями натрия. Снижение секреции альдостерона у видов птиц, живущих в среде с засоленной водой и употребляющих «соляную воду», следует рассматривать как биологическое приспособление надпочечников.

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Acta Biol. Hung., **15** (2), 181—190 (1964)

EXPERIMENTAL STIMULATION OF VASCULARIZATION OF BRAIN TISSUE IN CHICK EMBRYOS

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(Received March 25, 1964)

Synopsis

Author investigated the stimulating effect of complete adrenal extract on vascularization of the brain tissue of chick embryo explanted to chorioallantoic membrane. Performing more than 300 explantations a marked ingrowth of capillaries was observed if the brain grafts were pretreated with complete adrenal extract. Brain fragments from 3 to 21 day-old chick embryos and 1 to 10 day-old chicken were examined for normal vascularization. Explanting untreated brain pieces of chick embryos of various age to chorioallantoic membrane the degree of capillary ingrowth was investigated. Author found that the prosencephalons of 5 to 6 day-old chick embryos either in their original sites or explanted to chorioallantoic membrane, displayed a minimal ingrowth of capillaries, the planimetrically determined percentage ratio of the capillary cross-sections being below 1 per cent compared to the surface of the whole explant. On the other hand, if the brain grafts of 5 to 6 day-old chick embryos were pretreated before explantation with complete adrenal extract they exhibited a relatively dense capillary network within 4 days, which amounted to 6 to 7 per cent of the area of the explant. Pretreatment with complete adrenal extract seems to have a stimulating effect on vascular ingrowth in tissue grafts.

Introduction

The morphological aspect of the vascularization of different organs was studied by numerous authors but only a few recent data in literature refer to the possibility of prompting the organism to stimulate vascularization.

CHALKLEY, ALGIRE and MORRIS [1] made attempts to promote the process of vascular repair in experimental wounds of mice by administration of dietary proteins.

In connexion with vascularization the question arises whether the density of the capillary network and its structure can be changed and whether the organism can be prompted to form capillaries in a tissue which was unsupplied by capillaries up to that time, in other words whether we are able to induce capillary ingrowth in a tissue.

According to the experimental results of KISS and KROMPECHER [4] of the numerous substances tested by them the complete adrenal extract proved to be most effective in stimulating vascularization.

If untreated bradytrophic tissues (umbilical cord, developing cartilage) were explanted to chorioallantoic membrane the capillaries of the chorioallantoic membrane did not grow into these tissues. If these grafts had been pre-

treated with a solution of protoporphyrin (300 μg/ml), a slight ingrowth of capillaries was observed in the marginal areas of the bradytrophic tissue. On the other hand, if pieces of umbilical cord were pretreated with complete adrenal extract the ingrowth of capillaries from the chorioallantoic membrane was consistent and fairly demonstrable.

The vascular supply of circumscribed areas of the grafts was also examined. The homogenous transplants of spongious bone were found to become entirely destroyed and replaced by a new capillary network produced by the host organism within a few weeks. It is also known that in certain diseases (tuberculosis, tumors etc) the vascularization of the damaged tissue becomes poor or entirely disappears. Some pathological cases, e.g. rosacea, are known to be accompanied by a marked increase of capillaries. In paralysis progressiva the capillaries of the brain were found to increase in number [3].

MÜLLER [7] in his monograph dealing with the human capillary system gives a survey of the development of our knowledge concerning this question. MÜLLER points out the great variety of the capillary distribution depending on the topographical circumstances of the human body. In the literature concerning the development of capillaries the work of MÜLLER has an important place in describing the functional alterations of tissues in certain diseases from the viewpoint of capillary supply. Thus not only the structure of certain territories is described but also their functional changes.

The object of our present investigations was to study the effect of adrenal extract [4] on the capillarization of the developing brain of chick embryos explanted onto chorioallantoic membrane. These studies required the preliminary examination of the spontaneous vascularization of the developing brain and that of the vascularization of untreated brain grafts explanted onto chorioallantoic membrane.

Material and method

The method of explantation on chorioallantoic membrane is well known. In the last decades this method has been used by numerous authors for the study of tissue differentiation to approach and elucidate hereby certain chemical, biological and embryological problems [2, 8, 9]. Our investigations have been carried out in three groups.

(1) First the spontaneous vascularization of the brain of the chick embryo was examined. The brains (and a few spinal cords) of a total of 196 chick embryos (aged 4 to 21 days) and chicken (aged 1 to 10 days) were investigated using the common histological methods.The specimens were fixed in formol and Susa solution, embedded in paraffin and serially sectioned. The sections were stained with haematoxylin-eosin, haem. chromotrop, Azan, orcein, methyl-green orcein and toluidine blue. The sections were projected on scale paper and the cross-sections of the ingrown vessels were drawn round. The surfaces of the areas enclosed by these contours were calculated in mm^2 and their relation to the visual field was expressed in percentage ratio. Special attention was directed to the vascularization of the cortex.

(2) In the second group of our experiments the vascularization of the brain of the chick embryo was studied in the course of its explantation onto chorioallantoic membrane. A total of 94 explantations were carried out under sterile conditions. The explants were grafted on chorioallantoic membranes of hen eggs incubated for 9 to 10 days. The small portions

of brain and spinal cord were prepared under stereo-microscope, conditions being kept as sterile as practicable. They were kept in physiologic saline at 37°C until explantation. The eggs incubated for 9—10 days were selected by transillumination. The localization of the embryo was marked on the shell. After washing the eggs with petrol an area of about 2—3 cm^2 was painted with iodine and about 1 cm^2 of the shell and its membrane were removed, taking care not to injure the chorioallantoic membrane. The prosencephalic or telencephalic piece (sometimes a piece of spinal cord) was placed on the chorioallantoic membrane in the vicinity of a vessel visible also gross-anatomically. The opening was covered with a sterilized mica slide fixed to the shell with paraffin and the egg put back in the incubator (37°C). After various intervals (2, 3, 4, 6, 8 days) the eggs were opened and Susa solution dropped on the explants. After a fixation of 5 to 10 minutes the grafts were excised together with the chorioallantoic pieces to which they adhered and after fixation in Susa solution they were embedded in paraffin and serially sectioned. By previous fixation with Susa solution the material can be more easily removed since the chorio-allantoic membrane becomes more rigid by this treatment.

(3) Only after the evaluation of the I. and II. series of experiment could we start to study in the III. part of our experiment the effect of the complete adrenal extract on vascularization. The adrenal extract was prepared according to the method described by KISS and KROMPECHER [4] namely, both adrenal glands of a mature rat (or dog) were homogenized in a Potter tube adding 2 ml of physiologic saline. Half an hour later the homogenate was centrifuged (3000/min.) and the butter-coloured extract pipetted off and heated to 37°C. The tissue pieces which displayed before treatment no vascularization or a very poor one, were soaked for half to one hour in this extract. The control pieces were kept in physiological saline until explantation. A total of 56 cultures were made and the brain fragments pretreated in this way could be easily cultured on the chorioallantoic membrane.

Results

Observations on the spontaneous vascularization of the brain of the chick embryo

No capillaries were observed among the cells of the brain vesicle of the 4 day-old chick embryo. The prosencephalon of the 5-day-old chick embryo displayed a rapid development; very few or no capillaries were seen in the sections. The cross-sections of the capillaries represent 0.1—0.2 per cent of the area of the section (Fig. 3). The percentage ratio of the cross-sections of the capillaries in the prosencephalon at different ages is the following:

0.27 per cent in the 6 day-old chick embryo
0.72 per cent in the 7 day-old chick embryo
0.95 per cent in the 8 day-old chick embryo

In the chick embryo of 8 days the differentiation between the cortical and medullar layers is already visible. Subsequently, the vascularization of the cortex was more thoroughly examined.

The percentage ratio of the cross-sections of the capillaries in the cortex compared with the areas of the section was the following:

1.76 per cent in the 9 day-old chick embryo
2.86 per cent in the 10 day-old chick embryo (Fig. 1)
2.63 per cent in the 12 day-old chick embryo
2.77 per cent in the 14 day-old chick embryo

During further development the differentiation of the capillaries comes in the foreground. In the medullar layer of the brain of the 10-day-old chick highly developed arterioles are visible (Fig. 2). A few explantations of spinal cord grafts were performed, too. According to our experience the vascularization of the spinal cord proved to be poorer than that of the brain.

Observations on the degree of vascularization in brain grafts of chick embryos explanted to chorio-allantoic membrane

In the following the results of *4 day culturing* are given since the maximum of vascularization is reached by this time. In the prosencephalon of the *5 day-old chick embryo* a slight or no ingrowth of chorioallantoic capillaries was observed. The percentage ratio of the cross-section of the capillaries in the explanted telencephalic fragments in proportion to the territory was the following:

In the	5	day-old chick	embryo	0.2—0.3	per cent (Fig. 3)
,,	6	,,	,,	0.73	,,
,,	7	,,	,,	1.02	,,
,,	8	,,	,,	1.56	,,
,,	9	,,	,,	4.31	,,
,,	10	,,	,,	6.45	,, (Fig. 4)
,,	12	,,	,,	5.11	,,
,,	14	,,	,,	4.62	,,

Thus the chorioallantoic capillaries form a more dense network in the explanted telencephalic fragment than in the spontaneously vascularized areas within the organism. In the subsequent stages of development the percentage ratio of vascularization is rather constant. The telencephalon of a *9—10 day-old chick embryo* displayed a marked vascularization as early as in *48 hour-old cultures*, the capillary cross-section being 2.3 per cent of the surface.

Effect of adrenal extract on the vascularization of the developing brain of the chick embryo.

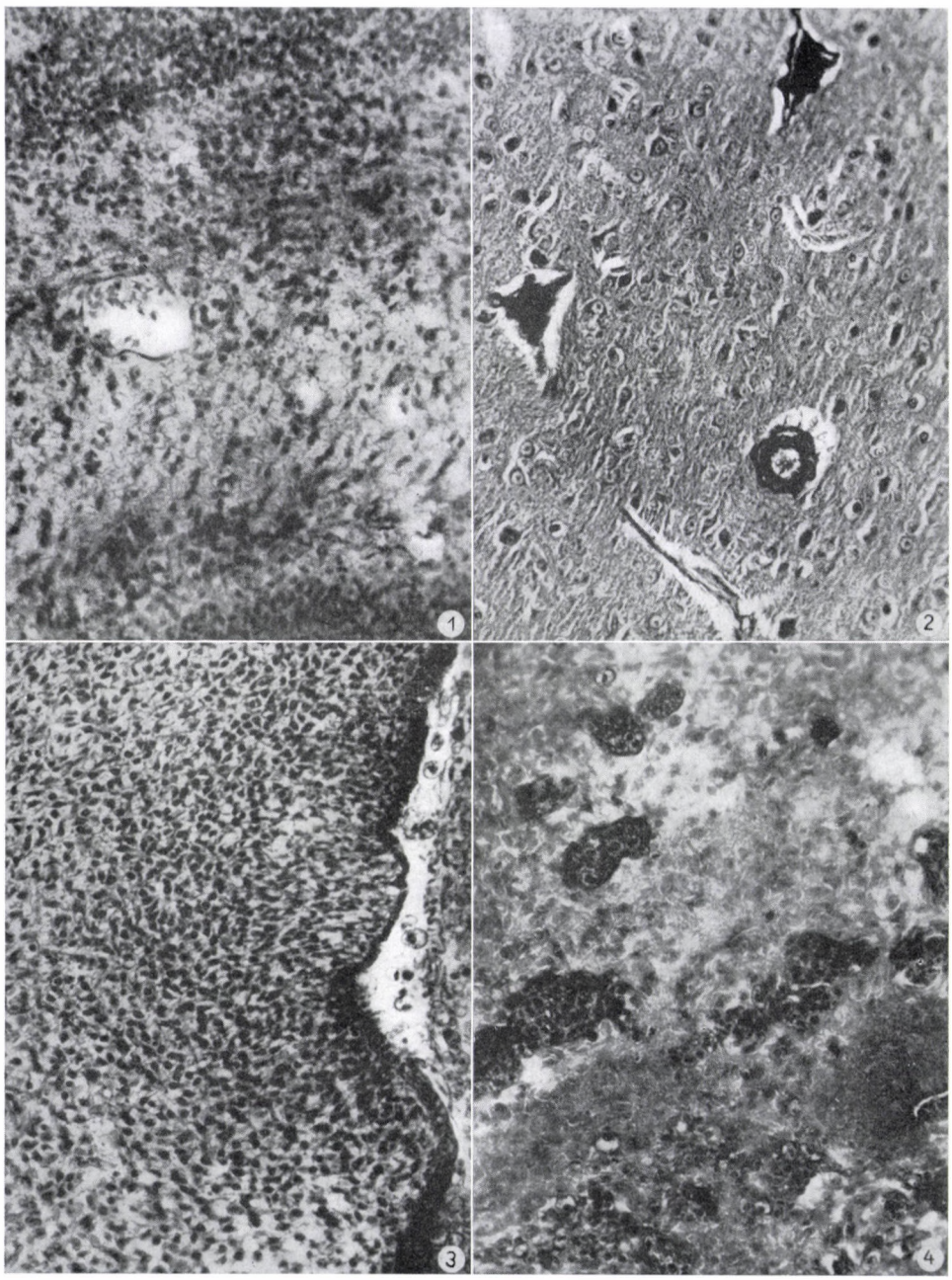
The question arose whether the adrenal extract can promote vascularization in the prosencephalon of 5—6 day-old chick embryos which are not vascularized or have a very poor vascular supply. It was found that as early as

Fig. 1. Telencephalon of 10 day-old chick embryo. The cross section of the capillaries occupy but a few per cent of the territory. Haematoxylin-eosin, × 200

Fig. 2. Brain of 10-day old chick. Marked arteriole and capillary supply. Azan, × 200

Fig. 3. Prosencephalon of 5 day-old embryo explanted onto chorioallantoic membrane cultured for 4 days. No capillaries are seen on the picture and in the other sections they were also very scarce. The cross section of the capillaries does not exceed 0.1—0.2 per cent of the territory. Azan, × 200

Fig. 4. Telencephalon of 10 day-old chick embryo explanted onto chorio-allantoic membrane cultured for 4 days. Vigorous capillary ingrowth. Methyl-green orcein, × 200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after 48 hours of culturing the capillary cross-sections of the prosencephalon of the 5½ day-old chick embyro amounted to 2.36 per cent of the territory, due to the effect of the adrenal extract (Fig. 5). In the 4-day-old cultures the percentage ratio of the capillary cross sections was found to be the highest: 6.15 per cent (Figs. 6 and 8). In 6—8 day-old cultures the degree of vascularization

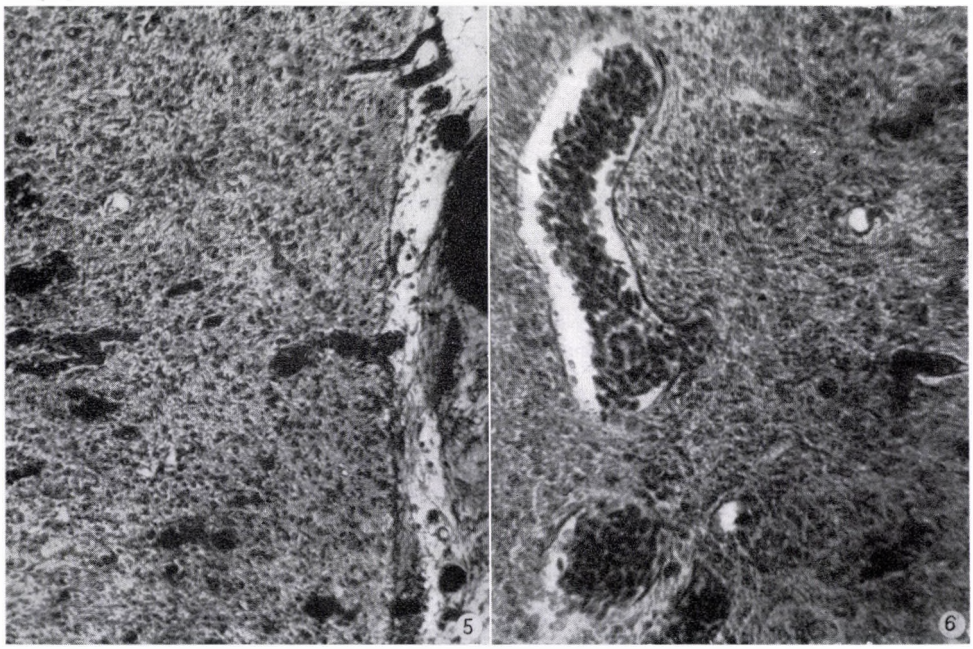

Fig. 5. Prosencephalon of 5½ day-old chick embryo pretreated with adrenal extract before explantation to the chorioallantoic membrane, cultured for 48 hours. The ingrowth of capillaries from the allantoic membrane is fairly visible in four sites. The winding capillaries have penetrated to about 500 microns high into the explant (compare with Fig. 4). Azan, × 200
Fig. 6. Same as in Fig. 7 in a 4-day-old culture. Capillarization reached its maximum. Azan, × 200

decreased, the capillary cross sections being 5 per cent. It is to be noted that the amount of capillaries of the chorioallantoic membrane of chick embryos older than 15 days showed a relative decrease. If the telencephalic fragments — which display a fairly good spontaneous vascularization — were treated with adrenal extract, the percentage ratio of their capillary cross section was the following:

In	7	day-old	chick	embryo	6.50 per cent
	8	,,	,,	,,	6.81 ,,
	9	,,	,,	,,	7.47 ,,
	10	,,	,,	,,	8.25 ,,
	12	,,	,,	,,	7.35 ,,
	14	,,	,,	,,	7.44 ,,

In the cortex of the 10-day-old chick embryo the percentage ratio of the capillary cross-sections amounted to more than 10 per cent (Fig. 7). In a few cases resorption of the grafts was observed both in the treated and untreated material.

Discussion

According to the conclusion of MÜLLER the development of the capillaries is dependent on hereditary conditions.

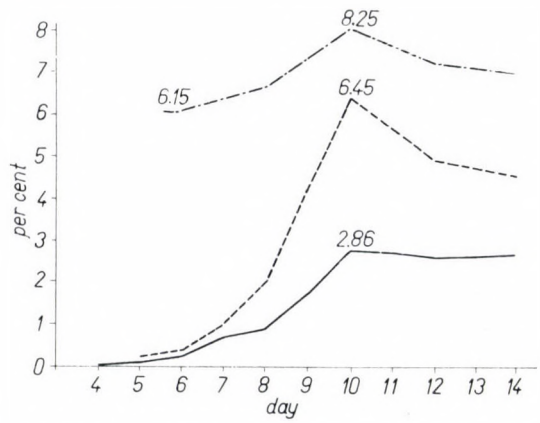

Fig. 7. Planimetric graph showing the vascularization of the brain of chick embryo. The abscissa indicates the age of the embryo and the ordinate gives the degree of vascularization in per cent. The vascularization reaches its maximum in the 10 day-old untreated chick embryo ——— spontaneous; — — — on chorioallantois; —. —. — after administration of adrenal extract (4 day-old explant). If the brain pieces were pretreated with adrenal extract the vascularization reached its maximum as early as at the age of 5—6 days. The cultures were all 4 days old

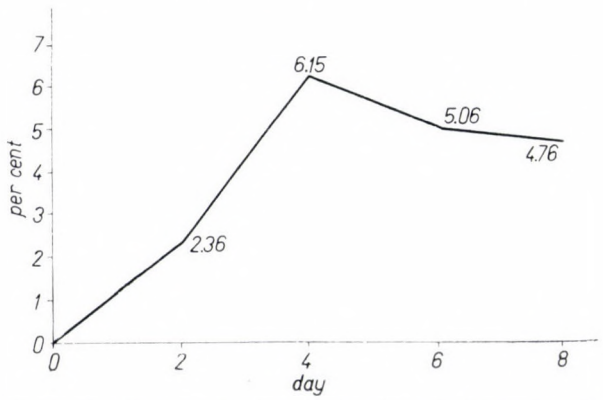

Fig. 8. Planimetric graph showing the vascularization of the brain of $5^1/_2$ day-old chick embryo pretreated with adrenal extract. Abscissa: duration of culturing; ordinate: the degree of vascularization in per cent. The vascularization reaches its maximum after 4 days of culturing

KROMPECHER and KISS [6] carried out experiments to investigate the role of causal factors of vascularization. WILLIAMS [11] believes that the proliferation of the endothelium and capillary development in autogenous transplantations is probably stimulated mainly by the degree and duration of hypoxia. It is however questionable how this opinion of WILLIAMS can be made conform with the appearance of mucopolysaccharides in areas where hypoxia is present. There are probably several promoting and inhibiting factors in vascularization.

In our experiments the increase of vascularization may be attributed to the effect of a complete organ, i. e. to that of the cortical and medullar layers of the adrenal gland. This finding, however, does not exclude the possible effect of a single chemical factor promoting or inhibiting vascularization, e.g. a certain capillary ingrowth was obtained in bradytrophic tissues explanted to chorioallantoic membrane following pretreatment with protoporphyrin [6]. Concerning the promoting factors of vascularization the following assumption seems to be obvious on the basis of the experiments above: the hormones of the pure complete adrenal extract may have a local effect on the graft and chorioallantoic membrane and a general effect on the whole chick embryo. From the viewpoint of vascularization the properties of following groups of hormones seem to be of interest:

1. Of the several effects of mineralocorticosteroids (desoxycorticoids) their stimulating effect on tissues is to be noted here.

2. The glycocorticosteroids (oxycorticosteroids) have in certain respects antagonistic effects. KROMPECHER and KISS [6] observed vascular pauperization in bradytrophic tissues treated with cortisone after their explantation to chorioallantoic membrane. KOSTENSZKY [5] found a decrease of capillarization in experimentally produced granulation tissue in the knee-joints of dogs in consequence of treatment with prednisolon. From the viewpoint of vascular ingrowth the glycocorticosteroids may have a decreasing effect on glycogenesis.

3. Experiments with androgenic and oestrogenic corticosteroids failed to give positive results as regards vascularization. Neither progesterone treatment could induce vascularization in bradytrophic tissues explanted to chorioallantoic membrane [6]. It is to be noted that the cortex of the adrenal gland contains also progesterone. However, the effect of adrenocorticoids in vascularization cannot be excluded.

4. The substances of the medullar layer of the adrenal glands may also have a role in the complex mechanism of vascularization. By the contractive effect of adrenalin and noradrenalin on the vessels these hormones can influence the blood pressure and blood distribution which may produce a certain change of environment for the endothelial cells. As it has been mentioned above, WILLIAMS [11] considers hypoxia to be the essential stimulus of endothelial proliferation and capillary development. The substances of the medullar

layer of the adrenal glands cannot be excluded as causal factors, on the contrary they can be made primarily responsible for the state of hypoxia. The primary endothelial growth and the development of arterial plexuses are not dependent on haemodynamic factors [10]. But that a state of hypoxia alone is not a sufficient stimulus to induce vascularization is proved by the fact that there are several bradytrophic tissues in the organism which are probably hypoxydotic and yet the capillaries do not grow into them.

Acknowledgement

The author is grateful to Professor St. Krompecher for his helpful advices and valuable criticism.

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EXPERIMENTELLE STIMULATION DER VASCULARISATION DES GEHIRNGEWEBES IN HÜHNCHENEMBRYONEN

Verfasser untersuchte die stimulierende Wirkung des kompletten Nebennierenextraktes auf die Vascularisation der auf die Chorioallantoismembran explantierten Gehirnstücke von Hühnerembryonen. Das Ergebnis von mehr als 300 Explantaten ergab, daß das Einwachsen von Kapillaren in die Gehirnexplantate durch eine Vorbehandlung mit Nebennierengesamtextrakt gefördert werden kann. Zum Vergleich wurde die normale Vascularisation des Gehirnes der Hühnerembryonen von 3—21 Tagen bzw. vom 1—10. Lebenstag der Hühnchen untersucht. Als Kontrolle für die Experimente dienten die unbehandelten Hirnexplantate der Hühnerembryonen auf der Chorioallantoismembran. Verfasser fand, daß die Kapillaren in die Hirnexplantate von 5—6 tägigen Hühnchenembryonen nur in einem minimalen Maße einwachsen. Ihr planimetrisch bestimmter Prozentsatz bleibt im Durchschnitt — auf das Gesamtterritorium des Explantates berechnet — unter 1%. Wenn aber ähnliche Hirnexplantate (von 5—6 Tage alten Hühnchenembryonen) vor der Explantation mit Nebennierengesamtextrakt

durchtränkt wurden, so entwickelte sich binnen 4 Tagen ein verhältnismäßig dichtes Kapillarnetz, das im Verhältnis zum Gesamtexplantat territorial 6—7% ausmachte. Eine Vorbehandlung der Hirnexplantate mit Nebennierengesamtextrakt scheint eine, die Vascularisation wesentlich fördernde Wirkung zu haben.

ЭКСПЕРИМЕНТАЛЬНОЕ СТИМУЛИРОВАНИЕ ВАСКУЛЯРИЗАЦИИ ГОЛОВНОГО МОЗГА ЗАРОДЫШЕЙ ЦЫПЛЯТ

Исследовалось действие общего экстракта надпочечников, способствующее васкуляризации, на тканевых культурах кусков головного мозга зародышей цыплят, эксплантированных на хориоаллантоидную оболочку. Проводились свыше 300 эксплантаций. Если куски головного мозга подверглись предварительной обработке общим экстрактом надпочечников, наблюдалась интенсивная васкуляризация. Автор исследовал на кусках головного мозга 3—21-дневных зародышей и 1—10-дневных цыплят без предварительной обработки нормальную васкуляризацию. Необрабатывавшиеся куски головного мозга, взятые из зародышей цыплят различного возраста, эксплантировались на хориоаллантоидную оболочку, и исследовалась степень васкуляризации тканевой культуры. Было установлено, что в переднем мозгу 5—6-дневных зародышей цыплят, независимо от того находятся ли они на первоначальном месте или в тканевой культуре на хориоаллантоидной оболочке, определенная планиметрическим способом доля поперечных сечений сосудов не достигает 1%, по сравнению со всей площадью тканевой культуры. В противоположность этому, если подвергнуть эти куски головного мозга зародышей цыплят предварительной обработке общим экстрактом недпочечников, то в пределах 4 дней в них проникает относительно более густая сосудистая сеть, охватывающая 6—7% общей площади. Предварительная обработка общим экстрактом надпочечников, по-видимому, пригодна для стимулирования васкуляризации тканевых культур.

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Acta Biol. Hung., **15** (2), 191—201 (1964)

EFFECT OF NEUTRAL RED ON MOUSE LIVER CELLS

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(Received April 20, 1964)

Synopsis

Neutral red is stored in the form of granules having a peribiliary distribution in mouse liver cells. The granules appear the faster the greater dose of the stain was administered. In the present experiments this time varied from 10 to 30 min. The neutral red granules first have similar number, size and intracellular localization as the lysosomes. In the first hour there is an almost uniform distribution of the dye in the different cells of the lobule, later a predominant accumulation in the centrolobular cells appears, where the size of the granules gradually increases. The granules remain smaller and their number is low in the peripheric cells. CCl_4 treatment produces a different localization. The dye accumulates in the peripheric cells and the centrolobular cells remain empty or contain but few granules. In the second hour an RNA containing substance (crinom) accumulates in the dye granules. This substance contains a PAS positive component. The pace of its appearance does not depend on the dose administered. Crinom formation is most intense in the centrolobular zone under normal conditions but in the peripheric cells after CCl_4 treatment. Dye granules containing this substance are more resistant to acetic acid treatment then early granules which are still free of it. Parallel with the appearance of neutral red granules the acid phosphatase activity of lysosomes markedly drops. Under normal conditions this is most marked in the centrolobular cells, after CCl_4 treatment in the peripheric ones. These results suggest a certain relationship between lysosomes and dye granules. Either the dye is accumulated in the lysosomes or the latter fuse with the forming dye granules while losing partly or completely their acid phosphatase activity.

Introduction

Vital dyes have been extensively used in the study of cell morphology and physiology since several decades. There are some scopes of problems where they are successfully applied even to date. One of these problems is the formation of crinom. Basophil substances, the so-called crinom, are known to accumulate in neutral red granules stored in the cytoplasm [3]. The basophily of the crinom granules is due to RNA and/or DNA [13, 30, 31, 40]. The mechanism of crinom formation is not known as yet.

Vital staining is a useful method to study the excited and resting state of the cell, further of cellular damages. It is known that the distribution of the vital dyes depends on the state of the cell and that the excited protoplasm has a markedly increased dye-binding property [18, 19]. The storage, possible break-down and elimination of substances entering the cells can also be studied using vital dyes.

These possibilities of the vital staining served as starting points in our experiments concerning the effect of neutral red on mouse liver cells. The following problems were studied: 1) The dependency of the staining of the liver cells on the amount of stain administered; the distribution of the dye in controls and after CCl_4 treatment; 2) The process of crinom formation in the above cases; 3) Stability of the dye granules; 4) The relationship of neutral red granules and lysosomes.

Material and method

The experiments were performed on 108 male albino mice of 22 to 28 g weight. One group received neutral red (Neutral red extra B, Light) intraperitoneally at a dosage rate of 0.125 to 1 mg dissolved in 1 ml distilled water. The animals were killed by decapitation 5 min to 24 hours after dye administration. Pieces excised from the liver were fixed in Helly's or Carnoy or in formol-sucrose [9].

In certain cases two injections were repeated in 24 hours. An other group received 0.04 ml carbontetrachlorid ientraperitoneally one and half hours prior to dye administration, a third group received carbontetrachloride after the dye. Controls remained untreated or were given 1 ml distilled water intraperitoneally at different times before decapitation. The distribution of neutral red was ascertained on frozen sections of the formalin fixed material. RNA was demonstrated with 0.1 per cent toluidine blue at pH 3.8. Parallel sections were digested with RNase prior to staining. Lysosomes were demonstrated either with the PAS reaction or on the basis of their acid phosphatase activity. For the latter formol-sucrose fixed frozen sections were stained according to a modified Gomori-method [1]. Incubation at 35° C for 30 min. Incubation medium was always freshly prepared [22]. Azo-dye methods were also used for the demonstration of acid phosphatase. The hexazotised pararosanilin method of BARKA [1] was tried but the reddish brown reaction product could not be easily distinguished from the neutral red granules. Good results were obtained with a medium containing naphtol-AS-BS-phosphate and freshly diazotised 4-amino-2,5-dimetoxybensanilide (Echtblau RR) at pH 5. Incubation time at room temperature 15 to 30 min. The blue violet reaction product gives a contrast with the neutral red granules. Nonspecific esterase was demonstrated by incubating the sections in an alphanaphtyl acetate-Echtblau RR solution at pH 7.2 for 1 min. In some cases the amount of dye bound by the cells was also determined by elution of the dye with acidified alcohol from tissue samples and measuring the extinction of the eluates [7].

Results

The injected neutral red appears in the form of granules in the cytoplasm of liver cells. The granules appear the faster the greater amount of dye was administered. After a dose of 0.125 to 0.25 mg/g it takes 30 to 40 min., after doses of 0.5 to 1 mg/g it takes only 10 to 15 min. till the peribiliary stain granules appear in the liver cell cytoplasm. The granules resemble in number, size and distribution the lysosomes and are well seen in material fixed in Helly or formol. The number and size of the granules is the same in all cells first but already one hour after dye administration the centrolobular cells contain granules which are greater than those in the peripheric cells. The difference between the two zones increases in time. 4 to 6 hours after injection the centrolobular cells are filled with large granules whereas the peripheric cells contain but a few small granules (Fig. 1). Later, in the 8th to 16th hour, a gradual decrease of

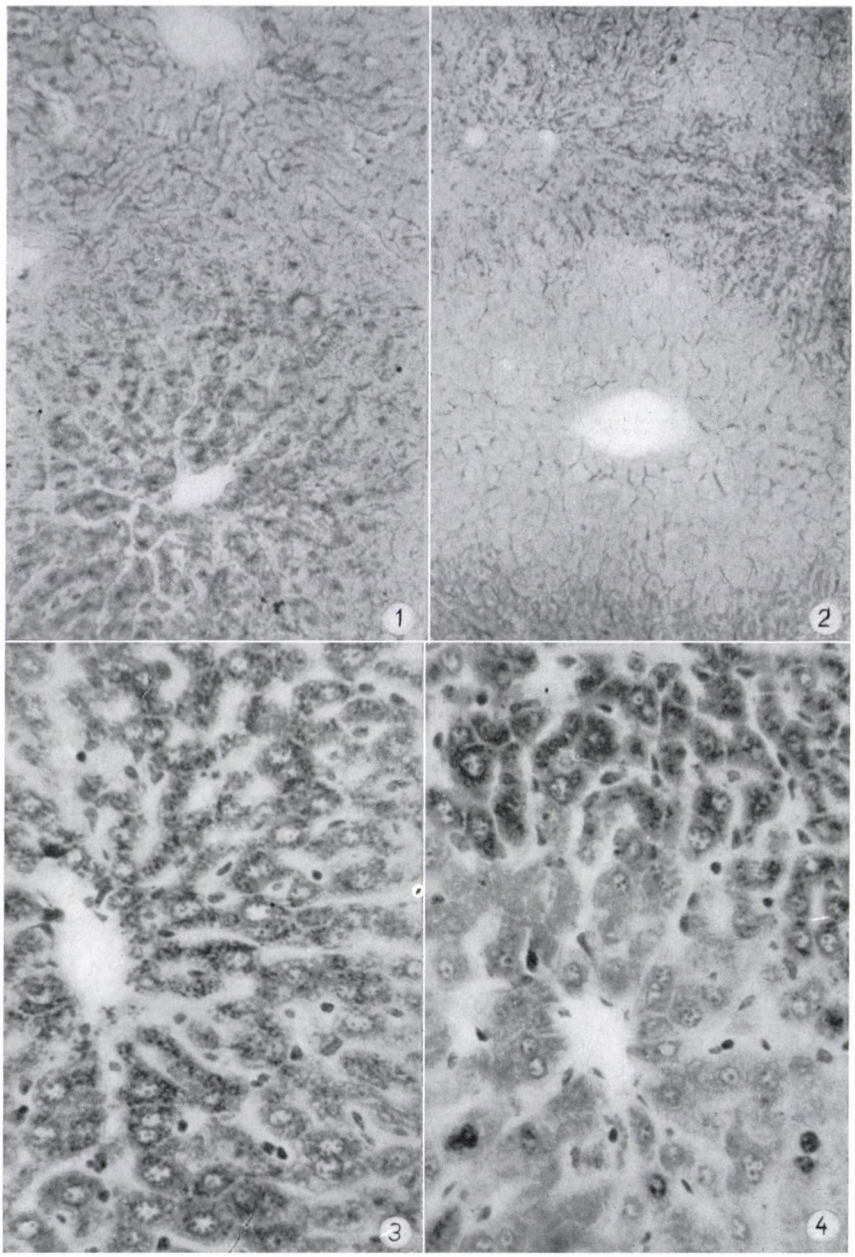

Fig. 1 Neutral red granules in the centrolobular cells 4 hours after dye administration (0.5 mg/g.). Formol-sucrose, × 150

Fig. 2. CCl_4 treated mouse. Neutral red granules in the peripheric cells 4 hours after dye administration (0.5 mg/g). Formol-sucrose × 150

Fig. 3. Crinom granules in the centrolobular cells 4 hours after administration of neutral red (0.5 mg/g). Helly, toluidine blue, × 400

Fig. 4. Liver of CCl_4 treated mouse 4 hours after the administration of neutral red (0.5 mg/g). Crinom granules in the peripheric cells. Helly, toluidine blue, × 400

the dye content commences. From the centrolobular cells the dye disappears approx. 24 hours after administration.

The liver of animals treated in advance with CCl_4 binds the same total amount of dye in the first hour of staining as the liver of controls. There is, however, a marked change in the distribution of the dye. In this case the centrolobular cells usually cannot store the dye in granular form and the greatest amount of the stain is found in the peripheral cells (Fig. 2).

Sometimes slight granule-formation may be found in the centrolobular cells, but the granules have a central situation near the nucleus instead of the usual peribiliar localization seen in normal liver.

Repeated neutral red injections may simulate the CCl_4 effect. If 0.125 mg/g dye was given, 24 hours later all neutral red is already eliminated from the liver but cells show signs of damage in the central zone. If dye is repeatedly given, no granules are formed in the damaged cells of the central zone although intense granule formation is observed in the cells of the intermediary and peripheric zones.

In the second hour after dye administration an intensely basophil substance appears on the surface of the granules (Crinom of Chlopin [3]). Ribonuclease digestion abolishes the basophily thus showing the RNA content of the crinom. In contrast to the speed of the neutral red granule formation which was found to be dose dependent, the crinom appeared in the same hour independent of the dose in the range used in the present study.

The crinom increases in size and increasing amount of RNA is accumulated in it as the dye granules become larger. This process is accompanied by a decrease of cytoplasmic basophily. As dye accumulation is most intense in the central zone of normal liver lobules the crinom formation is most intense here, too (Fig. 3). As a contrast, after CCl_4 treatment crinom granules are formed only in peripheric cells (Fig. 4). Similar picture is observed after repeated doses of the dye.

Accordingly the crinom substance is formed only where cells are capable to store the neutral red in granular form.

In addition to RNA the crinom contains a PAS positive substance, too. To demonstrate this substance, sections of formol or Helly fixed material were kept in glycerol for six days and subsequently in 70% ethanol containing 2% sulphuric acid for 1 hour. The neutral red is thus removed from the section and its red colour cannot be confused with the colour of the PAS staining. Sections thus treated showed PAS positive granules identical in size, shape and distribution with the crinom granules. The PAS positive substance is dissolved by Carnoy which points to its presumably lipid nature.

Since the composition of the dye granules changes in time due to the appearance of the crinom, changes in their stability were also assumed. This point was tested by using two methods. In the first case livers were removed

from animals killed at different times after dye administration. Thin slices were cut some of which were immediately fixed, others were kept prior to fixation in Ringer or in Ringer containing 0.01 n acetic acid for 30 min. Acetic acid treatment destroys the neutral red granules in liver cells of animals which were killed 30 to 60 min. after the dye administration whereas the granules persisted in liver tissue of animals stained for 2 to 4 hours. At the same time acetic acid produced severe damages to the cells.

In another experimental set CCl_4 was given the animals at different times after dye administration. One hour later the animals were killed and the liver fixed. Following a 30 to 60 min. staining the dye granules do not disappear from the central zone although the concentration of the dye stops under the effect of CCl_4 and a peripheric dye accumulation commences in the lobules. Thus a uniform distribution of the dye granules can be observed in different areas of the lobule. Following a 4 hour staining no changes are elicited in the granules and their distribution in spite of signs of damage in the central zone.

With Gomori acid phosphatase reaction the lysosomes of the liver cells, having a characteristic peribiliary localization, are easily demonstrated (Fig. 5). As mentioned, the neutral red granules have in the first hour of staining similar size, number and localization. In a preceding communication [8] it was reported that the acid phosphatase activity of the lysosomes decreases under the effect of neutral red. This point was tested in detail with two methods. As early as 30 min. after dye administration a marked decrease and later frequently a complete disappearance of the acid phosphatase activity (as shown by the Gomori method) was observed. The decrease is most marked in the centrolobular cells, i.e. in cells accumulating most of the dye (Fig. 6). Some activity is frequently found in the peripheric cells even if it completely disappeared from the centrolobular cells.

The azo-dye method also revealed the characteristic peribiliary localization of the phosphatase activity but resulted in a more diffuse staining. This method did not show decreased activity 30 to 60 min. after dye administration and it even appeared frequently greater than in the control sections, but later this effect could be demonstrated also with this method.

CCl_4 treatment produces a marked change in this picture. Decreased activity of acid phosphatase is observed now in the peripheric cells, where most of the stain is accumulated. Lysosomal activity shows no or only slight decrease but frequent change of localization in the centrolobular cells. The granules are seen as perinuclear groups (Fig. 7). As mentioned, sometimes the dye granules had similar distribution in the centrolobular cells after CCl_4 treatment.

The effect of neutral red on esterase activity was also tested. In untreated animals this activity is higher in the cells of the central zone than in the peripheric cells. Longer (8 to 24 hours) neutral red treatment led to some decrease

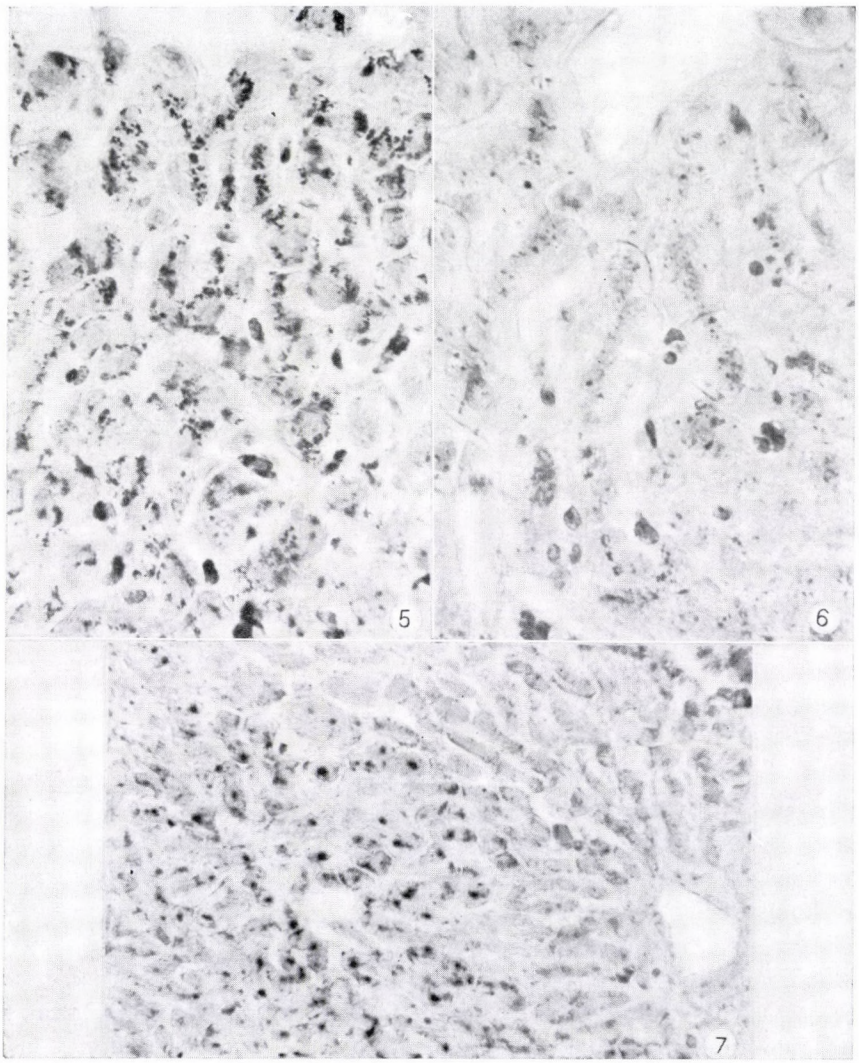

Fig. 5. Lysosomes in liver cells of a control animal. Formol-sucrose, Gomori reaction, × 400 *Fig. 6.* Centrolobular cells 4 hours after the administration of neutral red (0.5 mg/g). Formol-sucrose, Gomori reaction, × 425 *Fig. 7.* Liver of CCl_4 treated mouse 2 hours after the administration of neutral red (0.5 mg/g). Acid phosphatase activity in the centrolobular cells. Formol-sucrose, Gomori reaction, × 200

of this activity and to a frequent disappearance of differences between central and peripheric cells. In general the esterase activity did not exhibit as clear cut and sharp changes as the acid phosphatase activity. The use of E-600 [26] and NaF [33] for selective demonstration of the lysosomal esterase activity remained unsuccessful due to simultaneous blocking of both the lysosomal and cytoplasmic activity.

Discussion

Our data support the earlier reports on the accumulation of neutral red as peribiliary granules in the cytoplasm of liver cells [3, 17, 29, 38]. In these reports, however, no mention was made of the sharp differences in the neutral red stainability in different zones of the lobule. The heterogeneity of the cells within a lobule is well known [20, 23]. This heterogeneity demonstrated also in respect to the neutral red staining in an earlier report [8] and in the present communication can not be disregarded in studies concerning the effect of vital stains on liver cells. The present evidence demonstrates that the process of accumulation of dye granules can be controlled by certain treatments producing either a peripheric or a central deposition of the neutral red.

Nothing is known of the cause of the differences between cells in different zones of the lobule. Centrolobular cells cease to excrete stains (Na-fluoresceine) under hypoxia [27]. ZEIGER and WIEDE [41] found that acridine orange is excreted via the bile capillaries if present in small concentrations. Higher concentrations inhibited the excretion. It can be assumed on this basis that the peripheric cells, being under more favourable conditions, are able to excrete quickly the neutral red whereas the centrolobular cells excrete the dye slower, i.e. more dye enters their cytoplasm than leaves it, thus leading to increasing accumulation of neutral red. The stored dye itself inhibits subsequently the excretory mechanism producing still greater accumulation. This is how the difference between the central and peripheric zone arises. CCl_4 causes a damage of the central cells. As known, damaged cells are unable to accumulate vital stains in granular form [18, 19]. It can be assumed that the same process occurs in the peripheric cells in the latter case.

Several communications report on crinom formation in liver cells under prolonged effect of neutral red [3, 17, 30, 38]. This was confirmed in the present observations, too. It deserves special mentioning that in mouse liver cells there is an early commencement of crinom formation (in the second hour after the dye administration) further that the central and peripheric cells of the lobule show great difference also in this respect. The substance of the crinom contains nucleic acids (RNA and/or DNA) [8, 10, 13, 30, 31, 32, 40], the origin of which is not known. The RNA might originate from the ergastoplasm [32].

Electron microscope studies have shown the alterations due to neutral red to occur first in the ergastoplasm [39]. No unaequivocal data are available concerning the occurrence of other substances [8]. The present work has shown two main components to be present in the liver cell crinom — RNA and a PAS positive substance presumably of lipid nature. These can bind the dye and thus explain the increased stability of the dye granules after appearance of the crinom. SHTUTINA [36] and SHTEMPEL and TROSHINA [35] studied this

problem with similar methods but on different objects. The present work confirms their observations.

The conditions of the crinom formation are not well known. Since the crinom contains RNS it could be assumed that crinom would form easier in strongly basophilic cells than in others. However, KEDROWSKI [11, 12] demonstrated that no crinom is formed in the highly basophilic embryonal cells. SCHMIDT did not find any connection between the development of ergastoplasm and the intensity of crinom formation [30]. Present observations revealed that one prerequisite of crinom formation was the granular storage of the dye in the cytoplasm and the relatively long (in mouse liver one hour) persistence of the granules.

On account of the peribiliary localization of the neutral red granules it was assumed that the storage of the dye occurs in the Golgi apparatus [3, 7, 17, 29, 38]. In view of the recent lysosome concept [4, 5, 21, 22] a possible role of these organelles in the formation of neutral red granules might also be assumed. This possibility is mentioned also by BRACHET [2] and DE DUVE [4]. In electron microscope studies TANAKA [37] found that neutral red and other stains accumulated in special granules, in the segresomes. The latter structures are most propably identical to the lysosomes. According to KOENIG [14, 15] and OGAWA and co-workers [24, 25] the neutral red granules can be identified with the lysosomes on the basis of their localization, number and dimensions. Present authors arrived to a similar conclusion in an earlier study [8]. ROSENBAUM and WITTNER [28] mention the possible identity of the neutral red granules seen on the periphery of starved *Paramecium caudatum* with lysosomes. There are several points which support a certain role of lysosomes in the formation of neutral red granules: 1) In the first hour of staining the number, size and distribution of the neutral red granules is similar to those of the lysosomes (later they become larger). 2) The crinom substance is PAS positive. PAS positivity is a well known property of lysosomes [14, 15, 16, 21]. This staining can be demonstrated only after fixation in Helly or formaline in both structures. 3) The acid phosphatase activity of lysosomes diminishes parallel with the formation of neutral red granules. The decrease of activity is strongest where the granule formation is most intense. 4) No granular storage of dye can be observed in general in the centrolobular cells after CCl_4 treatment which produces a decreased stability of lysosomes [6, 34]. The two phenomena might have a connexion. In certain cases slight granule formation is observable in centrolobular cells also after CCl_4 treatment, but perinuclearily instead of a peribiliar localization. Under the same condition the lysosomes have a similar distribution (as shown by acid phosphatase or PAS staining). All these observations suggest that either the dye which entered the cell is accumulated in the lysosomes or that the lysosomes fuse with the neutral red granules.

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EINWIRKUNG VON NEUTRALROT AUF MÄUSELEBERZELLEN

Neutralrot wird in Form von peribiliären Granulen in den Leberzellen gespeichert. Das Erscheinen der Granulen im Cytoplasma erfolgt umso schneller, je größer die Menge des verabreichten Farbstoffes. Es erfolgte zwischen 10—30 Minuten unter den gegebenen Bedingungen. In der ersten Stunde haben die Granulen dieselbe Anzahl, Größe und intrazelluläre Verteilung, wie die Lysosomen. Während der ersten Stunde wird eine gleichmäßige Verteilung des Farbstoffes in den Zellen des Leberläppchens beobachtet, später wird es vorwiegend in den zentrolobulären Zellen gespeichert, wo auch die Körnchen immer größer werden. Sie bleiben kleiner und weniger zahlreich in den peripheren Zellen. Die Einwirkung von CCl_4 ruft eine Veränderung der Farbstoffverteilung hervor, die Neutralrotkörnchen erscheinen in den peripheren Zellen, während die zentralen Zellen leer bleiben oder nur wenige Granulen enthalten. Eine RNS enthaltende Substanz (Krinom) erscheint in den Farbstoffgranulen von der zweiten Stunde an. Sie enthält auch eine PJS positive Substanz. Die Erscheinungsgeschwindigkeit des Krinoms ist unabhängig von der Menge des verabreichten Farbstoffes. Die Krinombildung ist unter normalen Bedingungen in den zentralen, nach CCl_4 Behandlung in den peripheren Zellen die intensivste. In den mit Krinomsubstanz durchgetränkten Farbstoffgranulen ruft eine Essigsäurebehandlung geringere Veränderungen vor als in frühen, krinomfreien Körnchen. Gleichzeitig mit dem Erscheinen der Neutralrotgranulen vermindert sich die Aktivität der sauren Phosphatase in den Lysosomen und zwar unter normalen Bedingungen in den zentralen Zellen und nach CCl_4 Behandlung in den peripherischen Zellen. Diese Beobachtungen deuten auf eine Beziehung zwischen Lysosomen und Farbstoffgranulen. Der Farbstoff wird entweder direkt in den Lysosomen gespeichert, oder die Lysosomen verschmelzen mit den ausbildenden Körnchen unter vollkommenem oder partiellem Verlust ihrer sauren-Phosphataseaktivität.

ИЗУЧЕНИЕ ВЛИЯНИЯ НЕЙТРАЛЬНОГО КРАСНОГО НА ПЕЧЕНОЧНЫЕ КЛЕТКИ БЕЛЫХ МЫШЕЙ

Нейтральный красный отлагается в печеночных клетках мышей в виде гранул, сосредоточенных по ходу, желчных капилляров. Гранулы появляются тем быстрее, чем больше количество введенного красителя. В наших экспериментальных условиях они появились в течении 10—30 мин. Размер, локализация и число этих гранул в первое время такое же как у лизосом. В первый час краска распределялся в клетках печеночной дольки

более-менее равномерно, в дальнейшем однако она накопляется преимущественно в центральных клетках дольки, в которых размеры гранул постепенно увеличиваются. В периферических клетках гранулы остаются мелкими и относительно малочисленными. Под влиянием четыреххлористого углерода распределение нейтрального красного изменяется, краска накопляется преимущественно в периферических клетках, а центральные клетки дольки остаются пустыми или содержат только немногочисленные гранулы. Во втором часу после введения нейтрального красного в зернах красителя появляется вещество, содержащее РНК (так называемый кринoм *Хлопина*). Оно кроме РНК, содержит другой материал, дающий положительную ШИК реакцию. Быстрота появления кринома не зависит от количества введенного нейтрального красного. В нормальных условиях образование кринома происходит интенсивнее всего в клетках центральной части дольки, а после действия четыреххлористого углерода в периферических клетках. Более старые, пропитанные веществом кринома зерна красителя разрушаются под действием уксусной кислоты труднее, чем молодые, не содержащие кринoм зерна. Одновременно с появлением гранул нейтрального красного, активность кислой фосфатазы в лизосомах значительно снижается. В нормальных условиях это снижение сильнее всего выражено в центральных клетках дольки, а после действия четыреххлористого углерода в периферических клетках. На основании наших данных можно предполагать, что между лизосомами и зернами красителя существует связь. Краситель накопляется или непосредственно в лизосомах, или эти последние слиeаются с образующимися гранулами краски и при этом частично или полностью теряется активность кислой фосфатазы в них.

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Acta Biol. Hung., **15** (2), 203—211 (1964)

ELECTRON MICROSCOPIC LOCALIZATION OF THE "LEAD-REACTIVE SUBSTANCE" IN THE MYONEURAL JUNCTION*

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(Received May 20, 1964)

Synopsis

The fine structural localization of the lead-reactive substance has been studied in the myoneural junctions of the mouse intercostal muscle. It has been shown that lead combines with the postsynaptic membrane. It is suggested that the postsynaptic membrane consists of a mosaic-like pattern of the acetylcholine receptor, and, furthermore, that the postsynaptic membrane displays the role of a postsynaptic amplifier device of the action of presynaptically released acetylcholine. The peculiar micromorphology of the lead-reactive units of the postsynaptic membrane supports this assumption.

Introduction

It has been shown by SÁVAY and CSILLIK [11] that the myoneural junction has, in addition to an acetylcholinesterase activity, a conspicuous affinity for lead salts. Since the microscopic patterns obtained after lead nitrate fixation closely resemble those seen in sections stained for cholinesterase, it has been assumed that lead combined with the same submicroscopic structure that also contained cholinesterase activity. It has been shown, furthermore, that lead-treated samples are suitable for a polarization microscopic analysis that rendered possible a comparison of resting and active membranes in the molecular-anatomical sense of the word. It could be shown that synaptically released acetylcholine, by virtue of binding to membrane proteins, resulted in submicroscopic alterations of the lipoprotein framework of the membrane [4, 5].

Further histochemical studies revealed that not only lead but also other bivalent cations are preferentially bound to the postsynaptic membrane: in this respect, especially calcium appears to be of major importance [12].

Yet the question where this cation-reactive substance is actually located could not be answered either with histochemical or polarization microscopic investigations. Although LEHRER in some unpublished micrograms quite convincingly showed that the heavy electron-dense deposit of lead particles was located in the postsynaptic membrane complex, in 1961 ZACKS and

* Supported by a research grant (NB 00282-11) from the National Inst. of Neurological Diseases and Blindness, NIH., U. S. Public Health Service.

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BLUMBERG [13] suggested that lead was deposited not in the membrane but within the sarcoplasm of the finger-like expansions of the postsynaptic membrane. In view of the fact that the actual submicroscopic localization of the lead deposit is of crucial importance for the evaluation of the polarization optical studies, it was decided to re-investigate the electron microscopic localization of supravitally administered lead nitrate in the myoneural junction.

Methods

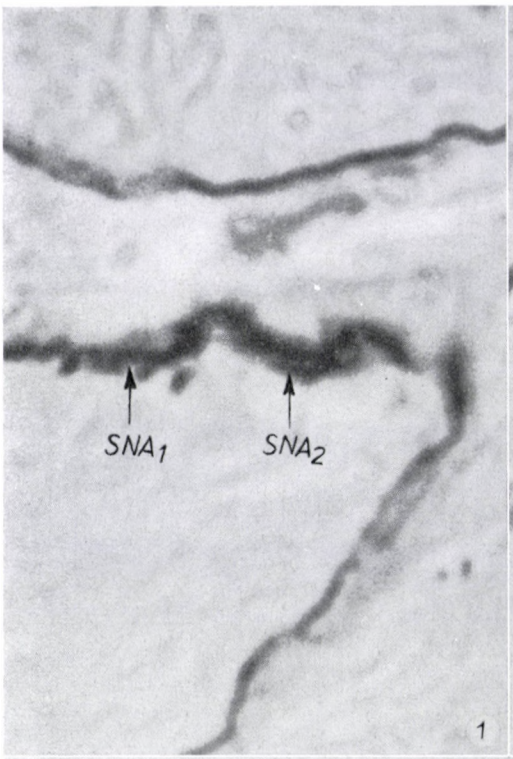
The investigations were performed on the intercostal muscles of adult mice. The animals were killed by decapitation, the thorax dissected and immersed immediately into a 0.1% lead nitrate solution, containing 10% formalin and sodium nitrate in sufficient amount to achieve isotonicity. The samples were kept in this solution for 5, 10, 20 and 30 minutes; blocks were cut from the intercostal muscles, fixed in osmic acid, dehydrated and embedded in Epon medium. Sections were cut on a Porter-Blum microtome; thick sections after treatment with yellow ammonium sulfide were examined under a phase contrast microscope; thin sections were examined and photographed in a Philips 100 B electron microscope. The best results were obtained in those samples kept in the lead nitrate solution for 20 minutes.

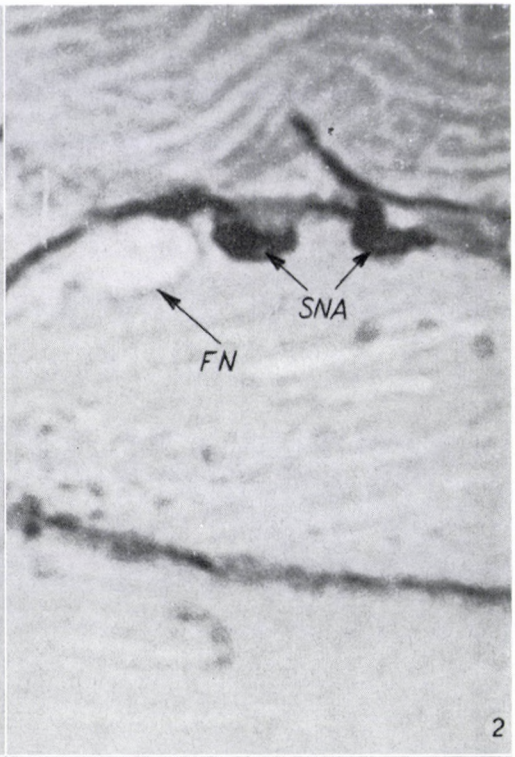
Results

In thick sections (Figs. 1 to 3) the surface membranes of the muscle fibres exhibited a heavy black staining in the vicinity of the end-plates. Surface areas more remote from the end-plates were unstained. The heaviest reaction occurred in the folded membrane-structure of the synaptic gutters constituting the subneural apparatus. In some of the sections, not only the semicircular cross-section of the gutter but also the individual folds were distinguishable. Thus there remains little doubt that in the postsynaptic membrane, both the actual subsynaptic and the neighbouring "para-synaptic" areas, are responsible for the lead binding.

The membrane-localization of the lead-reactive substance was even more conspicuous in thin sections. As seen in Figs. 4, 5 and 6, the heavy electron-dense lead deposit is localized in the surface membrane of the muscle; the reaction is more intense in the subsynaptic than in "para-synaptic" areas. Especially in the membrane areas lining the finger-like expansions of the subneural apparatus, a peculiar periodic arrangement of the lead-reactive membrane

Fig. 1. Thick section of a lead-treated intercostal muscle; phase contrast. *SNA* — heavy lead-reaction in the synaptic gutters of the subneural apparatus. Note the finger-like expansions at SNA_1. *Fig. 2.* Thick section of a lead-treated intercostal muscle; phase contrast. *SNA* — lead reaction in the gutters of the subneural apparatus. *FN* — fundamental nucleus. *Fig. 3.* Thick section of a lead-treated intercostal muscle; phase contrast. Numbers 1 —3 designate synaptic gutters. Note the fine structuration of the reaction at No. 3, due to the finger-like processes of the postsynaptic membrane


SNA1
SNA2
1


SNA
FN
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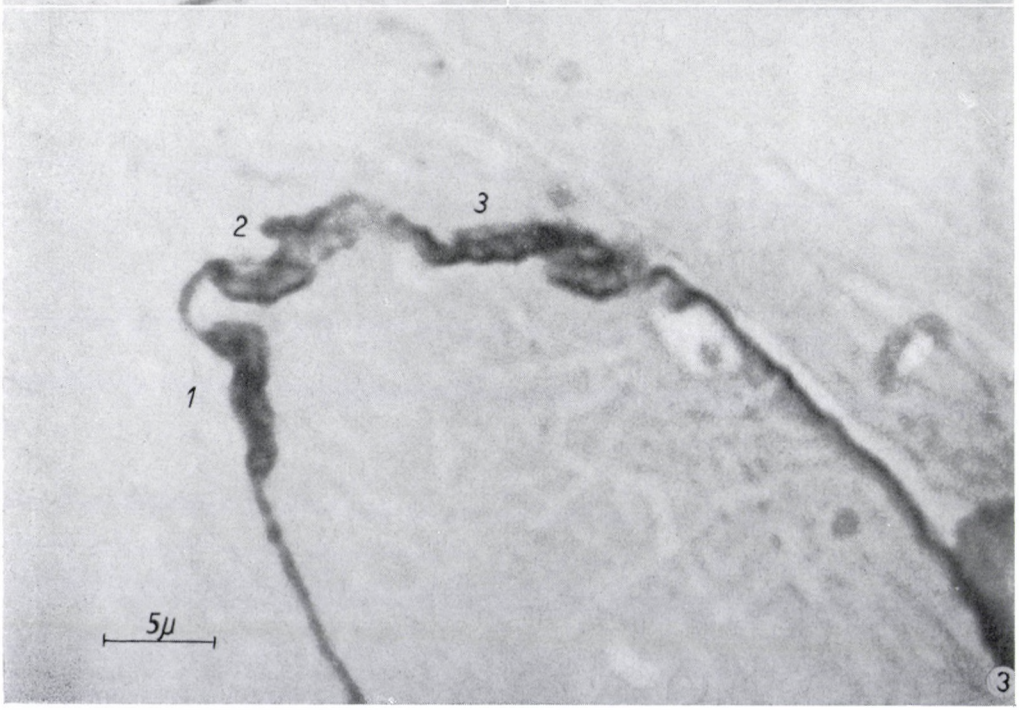
2
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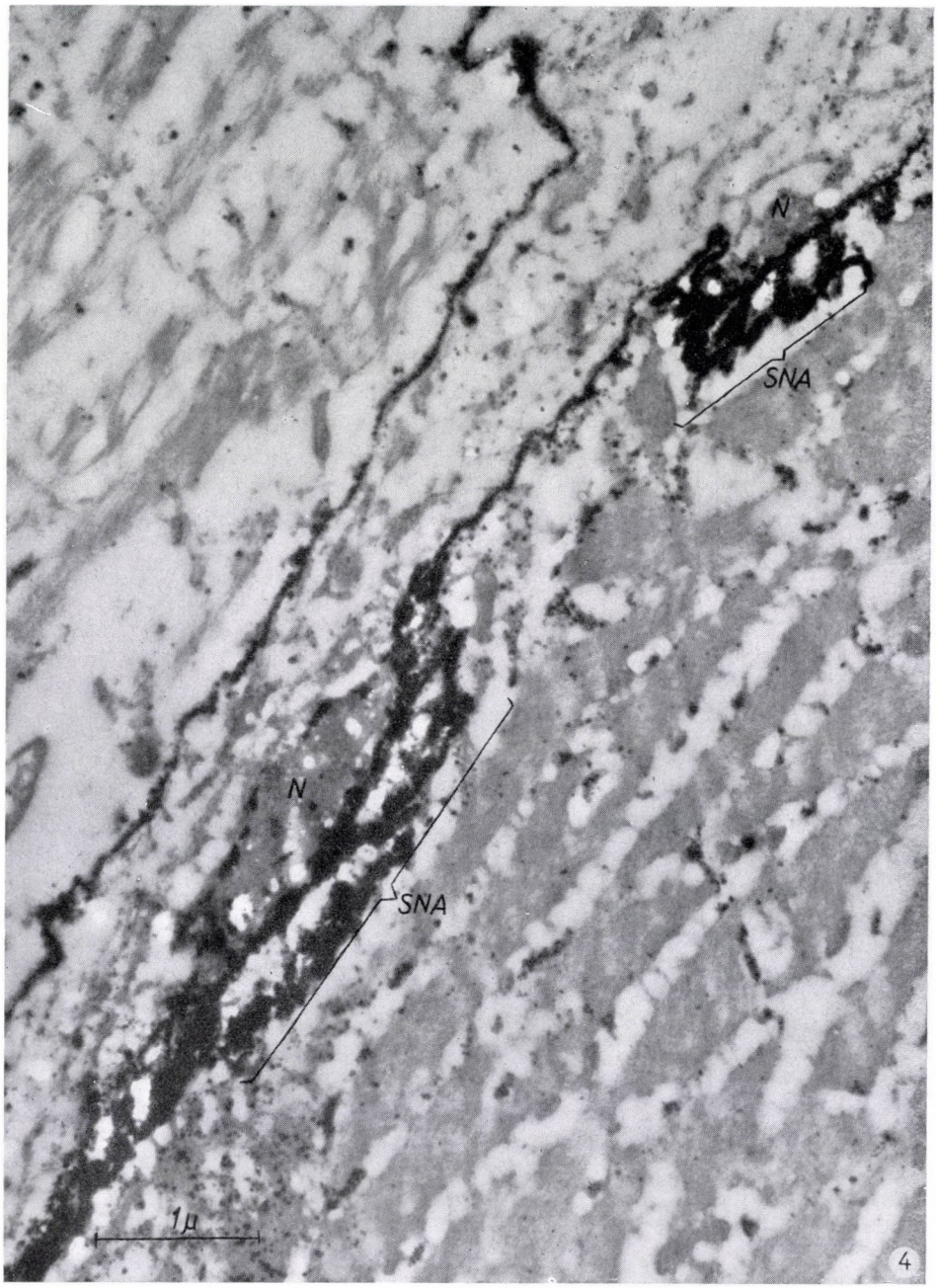

Fig. 4. Low-power electron micrograph of a lead-treated intercostal muscle. *N* — cross section of telodendrial motor nerve fiber. *SNA* — synaptic gutters of the subneural apparatus. Note the heavy lead reaction of the apparatuses and the much slighter activity of "parasynaptic" areas of the postsynaptic membrane

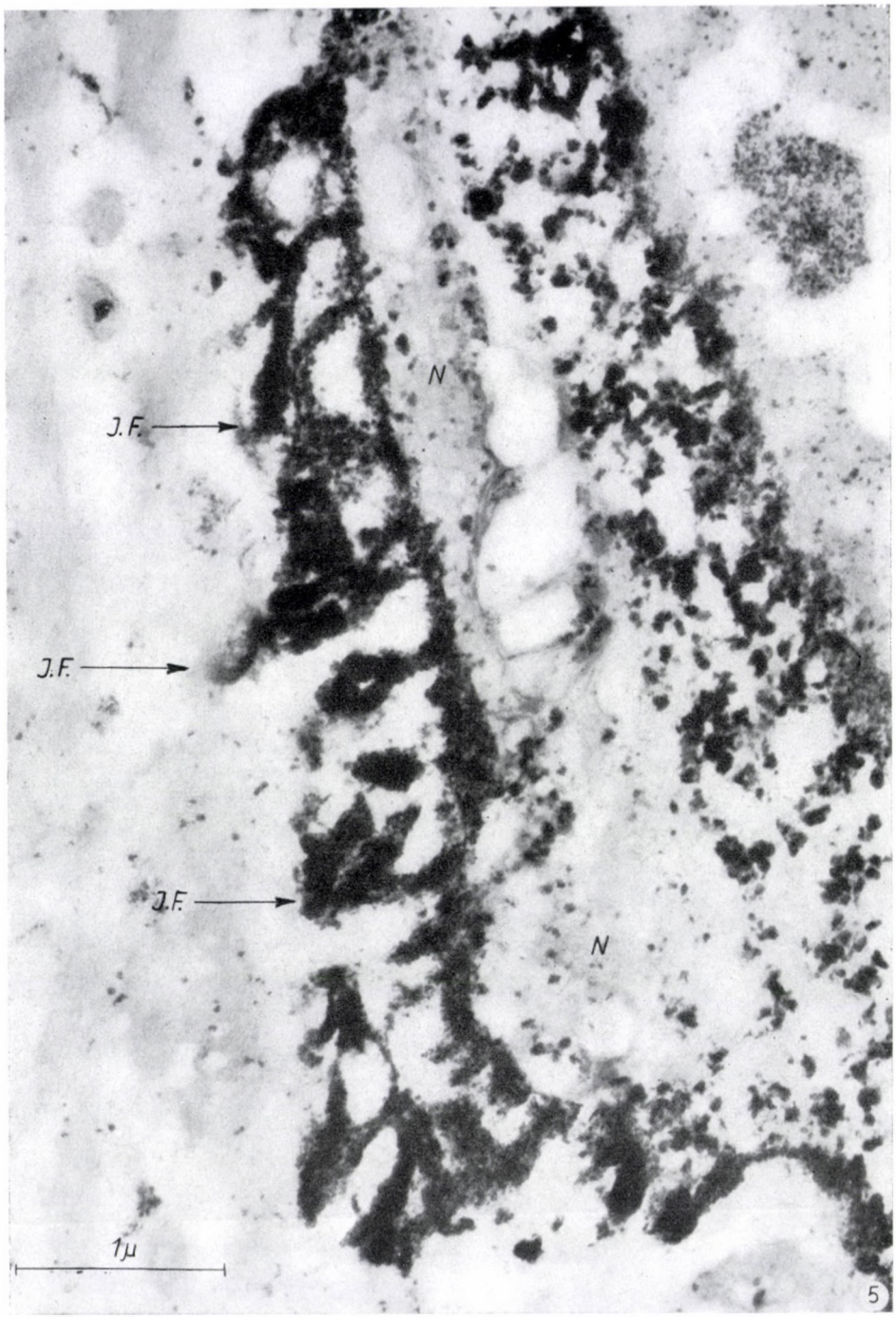

Fig. 5. Electron micrograph of a lead-treated myoneural junction. *N*—telodendrial nerve fiber; *JF*—junctional folds

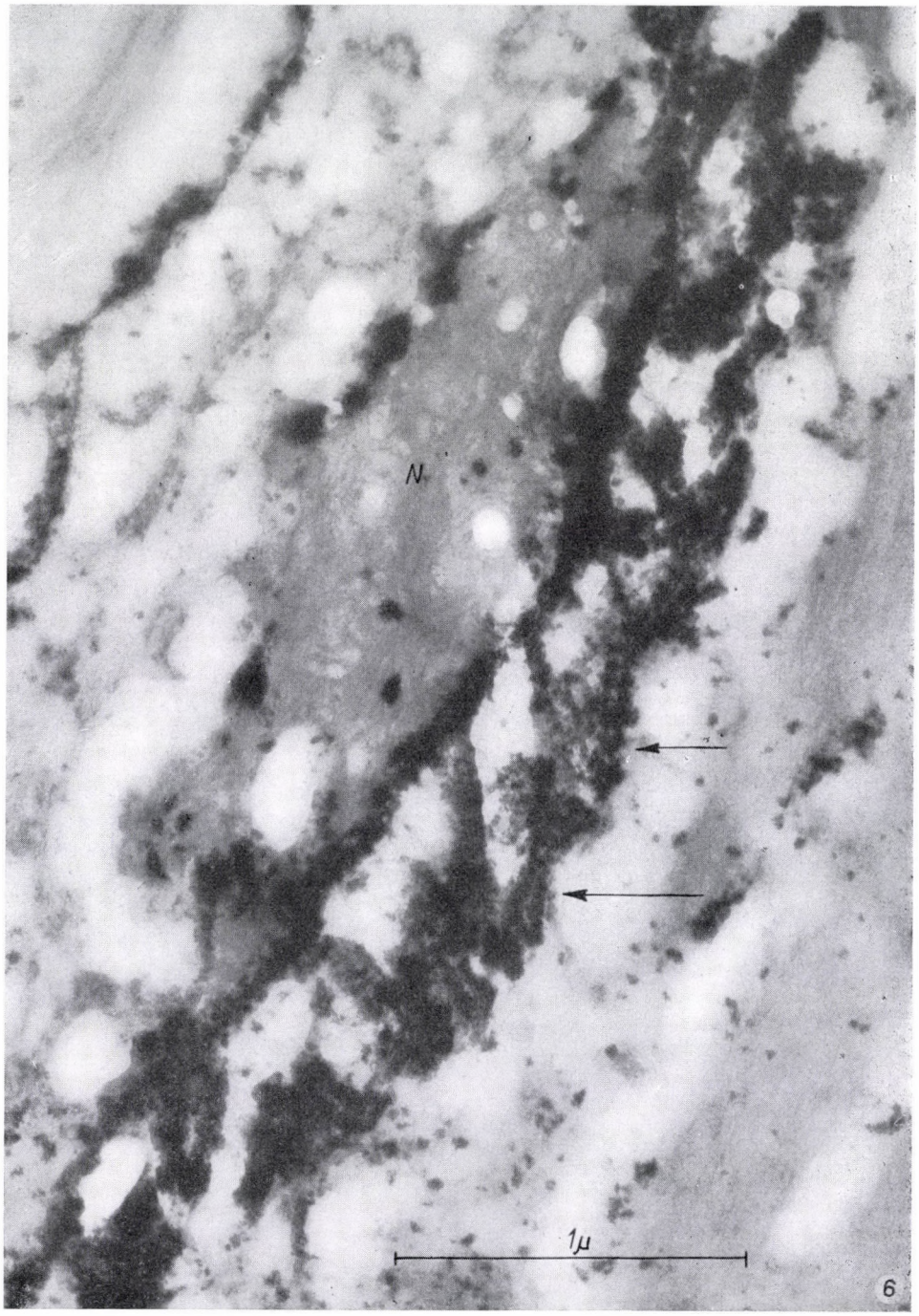

Fig. 6. High-power electron micrograph of a lead-treated myoneural junction. *N*—telodendrial nerve fiber. Note the periodicity of the lead reaction in the junctional folds and the lead-negative cores of the spherical bodies, constituting the periodicity (arrows)

material could be observed. The periodicity consists of roughly spherical bodies measuring 300—400 Å in diameter, isolated from each other by 100—200 Å interspaces. The periodic arrangement of the lead-reactive spherical bodies suggests that the entire membrane surface is made up of a mosaic-like pattern of lead-reactive units. Here and there, it also appears that the central parts ("cores", measuring 200—300 Å) of these spherical bodies are less electron-dense than their peripheral "shells".

Discussion

As already anticipated on the basis of light microscopic and polarization optical studies, the lead-reactive substance of the myoneural junction was actually found in the postsynaptic membrane. Therefore, the idea that the lead-reactive substance might be identical with the acetylcholine receptor — as suggested on the basis of the submicroscopic alterations after electrical and chemical stimulation [5] — is by no means in contradiction with the fine structural localization of this substance. However, there are two peculiar features of this submicroscopic organization, the importance of which cannot be overlooked. First of all, it appears that not only the "true" postsynaptic membrane (that is, the membrane compartment that actually underlies the telodendrial nerve fiber) but also the neighbouring parts of the muscle surface membrane have a lead-binding capacity. Therefore, if one accepts that the lead-binding membrane constituent is identical with the acetylcholine receptor — and we have good reason to believe that this is true — it has to be assumed that not only the actual postsynaptic membrane but also the neighbouring parts of the muscle surface membrane possess the property of answering with structural changes to the effect of acetylcholine. In other words, the acetylcholine-sensitive area appears to be significantly larger than the end-plate area itself. No appropriate measurements have been performed so far to establish the dimensions of the lead-reactive membrane areas, but, as a rough estimate, it appears that it surpasses the dimensions of the end-plate area by at least ten times or more. In this respect, it should be pointed out that, according to MILEDI's data [10], the acetylcholine-sensitive area in the rat diaphragm is about 0.5 mm, whereas the actual size of the end-plate measures 19—28μ [3].

The location of the lead-reactive material at the depths of the junctional folds also merits consideration. According to the calculations published by ECCLES and JAEGER [7], there is just enough time for presynaptically liberated acetylcholine-molecules to traverse the synaptic gap and reach the depths of the subsynaptic folds, often located as far as 0.7μ from the presynaptic terminal. Yet it is hard to believe that the extracellular space between the folds would allow any free diffusion for these acetylcholine molecules, as postulated by ECCLES and JAEGER, considering that it is filled by a gelatinous, electron-

dense material. Furthermore, it appears quite probable that acetylcholinesterase, associated with the inner surfaces of the folds [1,9,13] would hydrolyze all the acetylcholine long before it could reach the depths of the folds. Therefore, the lead-reactive material (or, in other words: the acetylcholine-receptor), located at the depths of the folds, could hardly respond with molecular distortions to presynaptically liberated acetylcholine molecules.

To overcome these difficulties, it has been proposed by one of us [6] that the folded postsynaptic membrane might play the role of an amplifier device, by releasing additional quanta of acetylcholine in response to that relatively small amount of presynaptically liberated acetylcholine which had diffused across the 500 Å synaptic gap. The main difficulty in accepting this suggestion is the apparent absence from the postsynaptic membrane of synaptic vesicles, repeatedly identified with the storage sites of acetylcholine. In this respect, it is tempting to speculate about the possible importance of the lead-negative "cores" of the spherical bodies in the membrane. Although it is perhaps a bold suggestion, the following working hypothesis appears for us acceptible: the spherical bodies, constituting the postsynaptic membrane, are to be looked upon as small reservoirs of acetylcholine. The ester might be located in the 200—300 Å lead-negative core of the body, in a bound state. The shell, on the other hand, reacting heavily with lead, consists of the acetylcholine-receptor. The entire body is embedded in a gelatinous material possessing acetylcholinesterase activity. Once the presynaptically liberated acetylcholine initiated ionic fluxes, these would activate the neighbouring spherical bodies, setting acetylcholine free, resulting in a molecular distorsion of the receptor protein-shell of the bodies, thus establishing a gradually expanding depolarized area.

The idea of a postsynaptic amplification of presynaptically liberated acetylcholine, supported by the heavy acetylcholinesterase-reaction of the postsynaptic membrane as well as by the peculiar micromorphology of the lead-reactive acetylcholine receptor, is actually an adaptation of KOELLE's concept [8] on the general neurohumoral role of acetylcholine. According to KOELLE, in sympathetic ganglia there occurs a presynaptic amplification of acetylcholine, whereas, in the postganglionic adrenergic terminals, acetylcholine triggers the release of norepinephrine, in a manner similar to that previously suggested by BURN and RAND [2]. Further details on the possible significance of a postsynaptic amplification in the myoneural junction, with special regard to the pathologic alterations of the junction in myasthenia gravis and nerve degeneration, will be presented elsewhere [6].

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ELEKTRONENMIKROSKOPISCHE LOKALISATION DES »BLEI-REAKTIVEN MATERIALS« IN DER MYONEURALEN JUNKTION

Mittels lichtmikroskopischer und elektronenmikroskopischer Untersuchungen wurde festgestellt, daß die »Blei-Affinität« der myoneuralen Junktion durch eine spezifische Ablagerung von Blei-Ionen in der postsynaptischen und parasynaptischen Zellmembran der quergestreiften Muskelfaser zustande kommt. Topographische und mikrostrukturelle Eigentümlichkeiten der Bleireaktion lassen vermuten, daß die postsynaptische Zellmembran die Wirkung des präsynaptisch freigesetzten Azetylcholins zu amplifizieren vermag.

ЭЛЕКТРОННОМИКРОСКОПИЧЕСКАЯ ЛОКАЛИЗАЦИЯ СВИНЦОВО-РЕАКТИВНОЙ СУБСТАНЦИИ В НЕЙРОМЫШЕЧНЫХ БЛЯШКАХ

Изучалась ультраструктуральная локализация свинцово-реактивной субстанции в нейромышечных бляшках межреберных мышц мышей. Выяснилось, что свинец связывается с постсинаптической мембраной. Оказывается, что постсинаптическая мембрана состоит из мозаикоподобно расположенных ацетилхолинных рецепторов, и далее, что она играет роль как усилительный прибор в действии пресинаптически освобожденного ацетилхолина. Своеобразная микроморфология свинцово-реактивных единиц постсинаптической мембраны подтверждает это предположение.

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Acta Biol. Hung., **15** (2), 213—230 (1964)

ELECTRON MICROSCOPE STUDIES ON DEVELOPMENTAL DIFFERENTIATION OF CILIARY GANGLION SYNAPSES IN THE CHICK

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(Received June 15, 1964)

Synopsis

Structural changes occurring in the characteristic calyciform synapses of the chick ciliary ganglion during the first few days after hatching have been studied under the electron microscope. In the newly hatched chick the synaptic terminal of the preganglionic fibres is a solid calyx. Subdivision of the calyx into individual axon-terminals begins immediately after hatching. Cleavage of the synaptic cup is effected by clefts cutting in secondarily from the contact surface between cell body and presynaptic cup. Some of the clefts are invaded by small irregular processes — so called "pseudodendrites" — of the cell body of the initial axonal segment. Desmosome type contacts between pre- and postsynaptic membranes lacking, however, specific aggregations of presynaptic vesicles are observed in the first few days. Transformation of these structurally "non-polarized" contacts into true synaptic attachments with specific accumulation of synaptic vesicles, occurs mainly between the 5th—14th days after hatching. This specialization of the contacts starts from the axon hillock-region and later proceeds to the periphery of the calyx. The whole synaptic arrangement, including the presynaptic calyx and the postsynaptic nerve cell body, is enveloped and isolated from the environment by a "loose myelin" structure formed by thin processes of the Schwann cells. A considerable extracellular space between pre- and postsynaptic elements is found mainly in the young ganglia, its amount being significantly reduced with advancing age. The possible functional significance of these structural changes in the developing ciliary ganglion is discussed in the light of recent observations on simultaneous changes of impulse transmission.

Introduction

The junction in the ciliary ganglion in birds and reptiles is both histologically and physiologically a remarkable subject the contacts being arranged in strictly one-to-one proportion of preganglionic and postganglionic fibres, and being effected by large calyciform synapses [8]. The synaptic calyx might cover about 25% of the unipolar ganglion cell surface, including always the axon hillock and an initial part of the axon [14].

The synapse described by DE LORENZO [2] under the electron microscope as a solid calyx in the chick embryo or the newly hatched chick, is according to SZENTÁGOTHAI [14] in reality a cup of densely packed individual presynaptic terminals in the adult, which originate from the same preganglionic fibre in brush-like manner. Strange irregular processes of the cell body and of the initial part of the axon — called "pseudo-dendrites" by SZENTÁGOTHAI [14] — invade the clefts between the individual terminal branches of the cup and establish

numerous synaptic contacts everywhere in this intricate labyrinth of the presynaptic cup, bringing about an excessive enlargement of the total synaptic contact area. A powerful specific ChE activity of this synapse has been reported [15] already much earlier. It was therefore all the more surprising that recently MARTIN and PILAR [11] reported an initially mixed chemical and electric transmission mechanism in the ciliary ganglion of newly hatched chicks, which becomes gradually converted into a purely electric one between the 5th — 28th days after hatching. With respect to these electro-physiological data it appeared worth while to try to correlate the functional changes from an initially partly chemical transmission mechanism into a purely electric one with developmental changes that must be supposed to occur during the same period of life.

Material and methods

Ciliary ganglia from chicks aged from 1 to 13 days after hatching and 2 to 3 months old ones have been used in these investigations. Ganglia were fixed in buffered and chilled 1 per cent osmic acid for 1 hour, then dehydrated in graded alcohol and embedded to Epon [9]. Ultrathin sections were made in an LKB-Ultratome and picked up from water on bare grids (300 mesh) without supporting film. Specimens were afterstained with lead citrate [12]. Observations were made in a JEM 5 Y electron microscope. Photographs were taken at original magnifications of 2000 to 16000.

Observations

The individual ganglion cellular complexes, each consisting of a ganglion cell, its synaptic apparatus and a Schwann cellular envelope are separated from each other by large connective tissue spaces (CTS), surrounded by which presynaptic as well as postsynaptic fibres may reach or leave their respective complexes. Each ganglion cell complex is enveloped by a capsule of Schwann cells (Fig. 1). The capsule (Figs. 4 and 6) shows several layers of double cell membranes, which run fairly parallel to the surface of the ganglion cell synaptic cup complex in regions devoid of nuclei but becomes highly irregular in the neighbourhood of the Schwann cell nuclei. Especially in the adult this layered membrane structure strongly resembles some myelin-like formation, particularly the loose myelin described by ROSENBLUTH and PALAY in the VIII-th cranial nerve ganglion of the goldfish [13]. A mechanism similar to the theory of myelin formation [5] would be quite acceptable as an explanation of the development of this structure — as also proposed by ROSENBLUTH and PALAY [13] for the VIII-th nerve ganglia, if it were not for the lack of mesaxons. From a careful investigation of this structure in newly hatched chicks, where the number of double lamellae is still rather small (Fig. 6) it emerges that the Schwann capsule in reality is built up of closely interdigitating parallel cell lamellae. Thus this structure is rather similar to the cellular sheaths of the

invertebrate tunicated peripheric nerve fibres as termed by EDWARDS and associates [4] and described in detail by ourselves [7]. During the later development, mainly from the 7th day after hatching onwards the cell lamellae become gradually thinner and more densely packed, so that the structure becomes almost completely myelin-like (Fig. 18).

The preganglionic fibre perforates the Schwann capsule at the site where the postganglionic axon leaves. It immediately widens into a synaptic calyx, that envelopes about 25% of the essentially adendritic ganglion cell surface. It is always the axonal pole of the ganglion cell that is covered by the calyx (Figs. 1 and 10) so that the postganglionic axon has always to pass through the presynaptic cup. In this respect we have to contradict the description of DE LORENZO [2], the situation having been originally correctly described by LENHOSSÉK [8] on the light microscope and by SZENTÁGOTHAI [14] on the electron microscope level. The presynaptic calyx, however, is a solid cup only during the first few days after hatching (Figs. 1 and 4). From the fifth day onwards the original calyx breaks up gradually into a multitude of individual branches of the same preganglionic fibre, which, however, still remain packed closely together into a cup-shaped arrangement. This subdivision of the original calyx begins with the outgrowth of blunt processes from the cell body around the 5th day after hatching (Fig. 8) which begin to impress themselves in invaginations of the presynaptic calyx. This process is already clearly advanced on the 7th day (Figs. 12 and 13) and is accompanied by the development of extracellular space between the original contact surfaces of the calyx (Figs. 12 to 14), just as between axonic profiles (Fig. 9). There is also another process involved in the subdivision of the presynaptic calyx. As seen in Figs. 12 and 14, there are some profiles completely surrounded by a double membrane and containing synaptic vesicles. This picture can be interpreted only by assuming that, independently of the intrusion of cell processes, the presynaptic membrane begins to invaginate in the form of double layered cylinders. By the 9th day after hatching these two processes have changed the presynaptic cup into an intricate labyrinth of closely packed and mutually invaginated profiles with an occasional cell process in between (Fig. 14). On the 10th and 13th days the development of the cell processes is already in advanced stage (Fig. 16), their structure and relations to the presynaptic elements are settled, however, in final manner only in the adult (Fig. 17). These cell processes have been described already by SZENTÁGOTHAI [14] and have been called "pseudodendrites". This term appears to be justified when one considers their late development, their short albeit sometimes branched course and especially the complete lack of any definite arborization pattern; an almost rigidly determined branching pattern being otherwise characteristic for all true dendrites. Additionally these pseudodendrites originate not only from the axon hillock but also very regularly from the initial segment of the axon (Figs. 10 and 11).

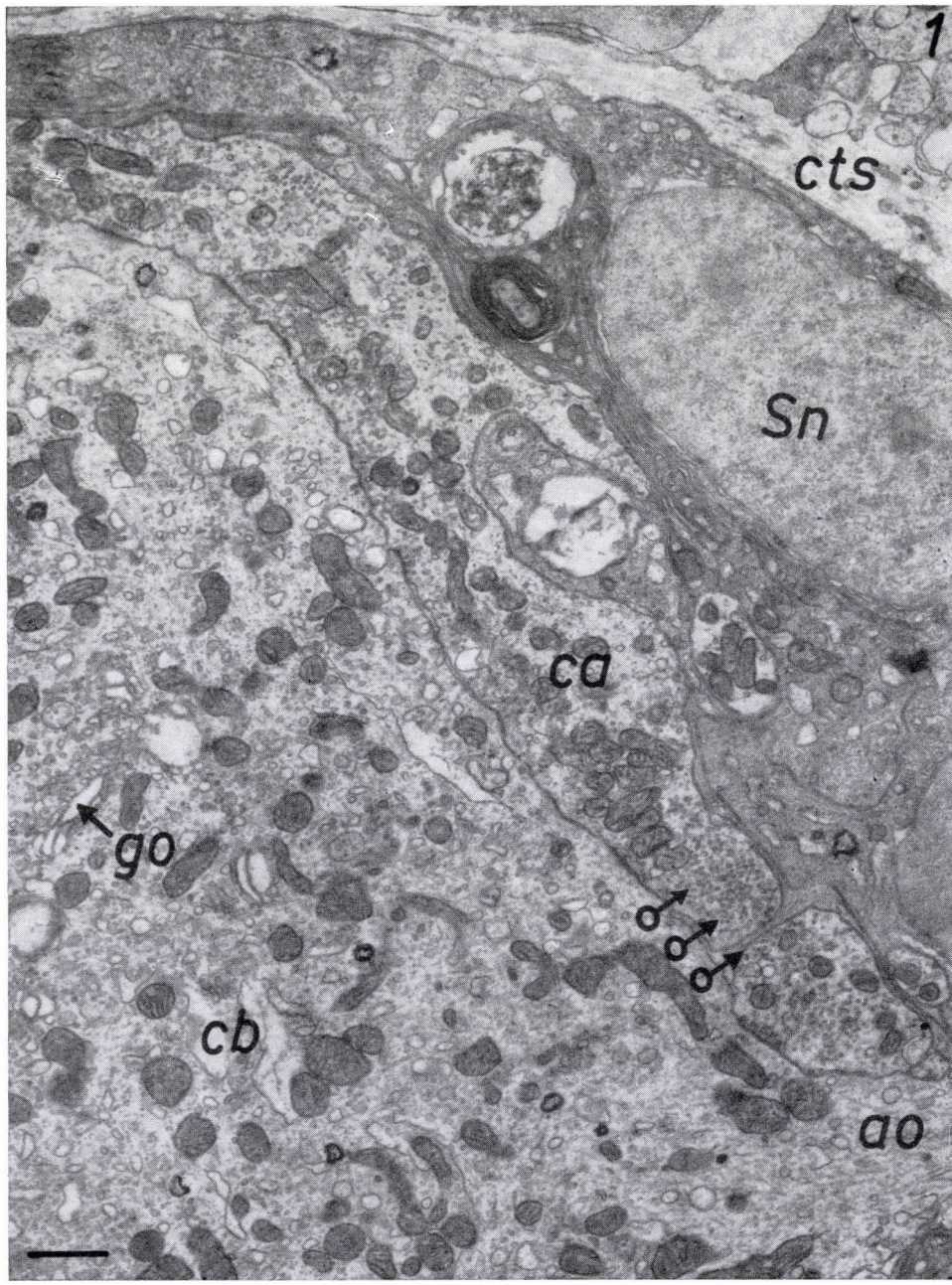
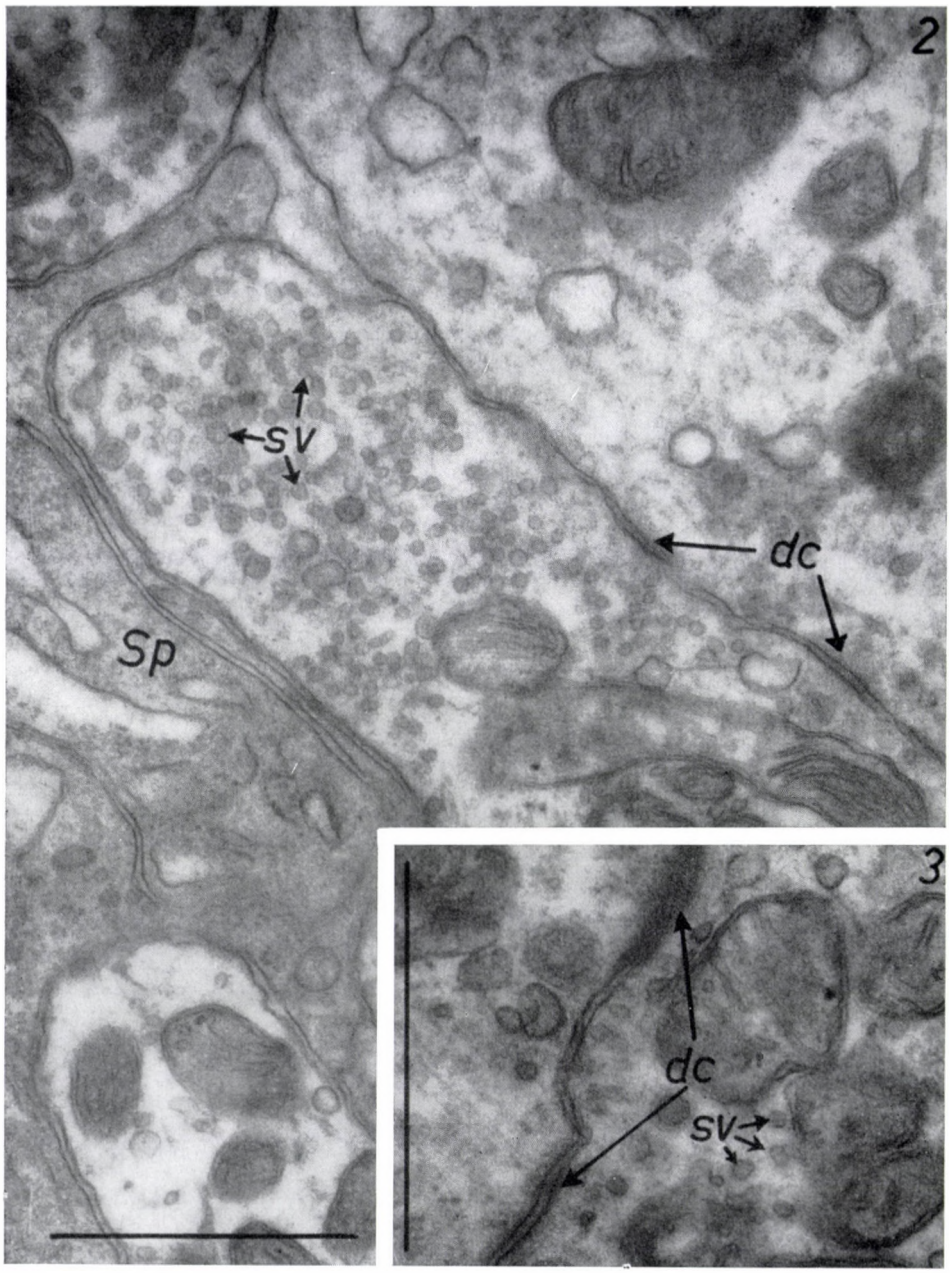
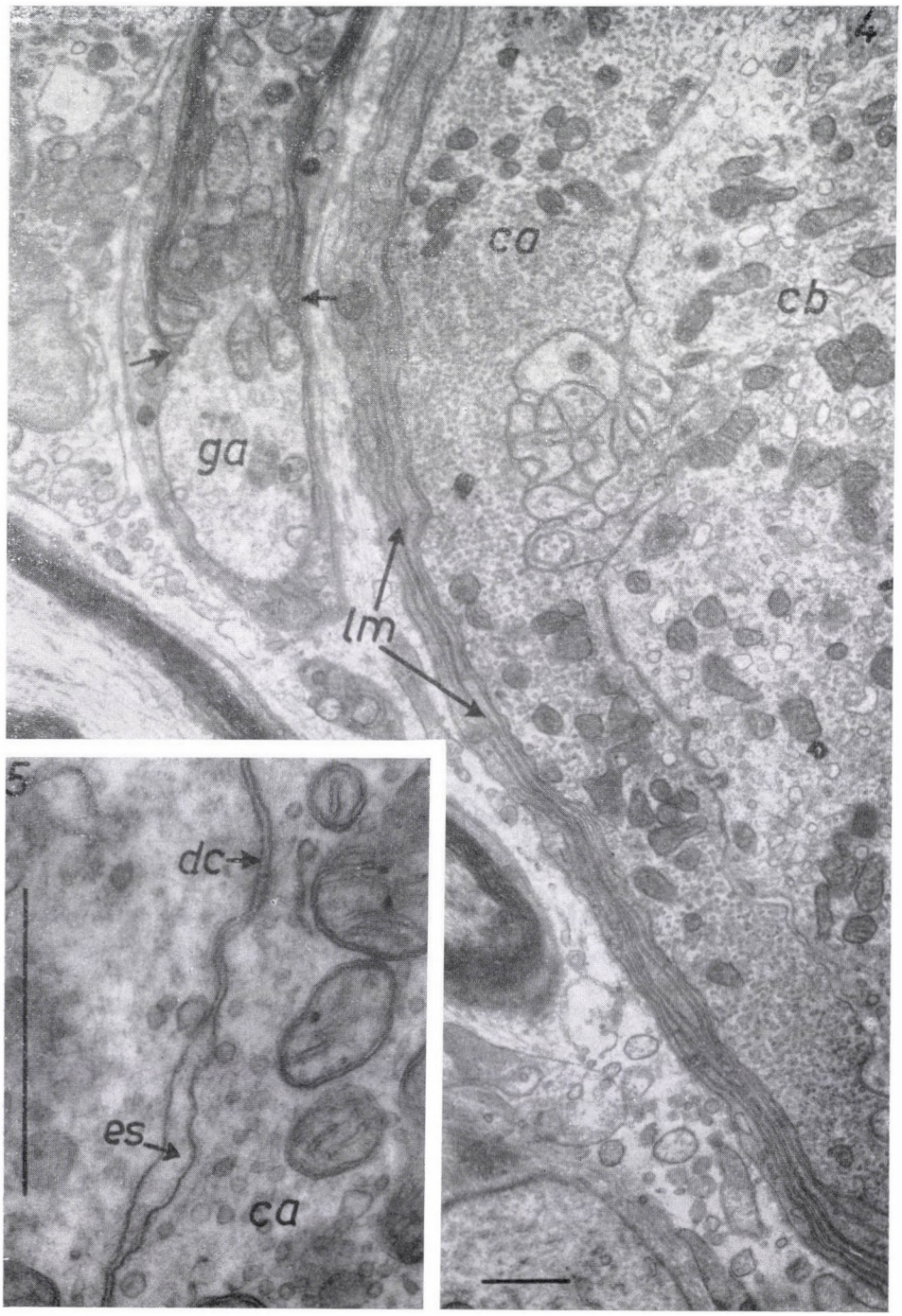


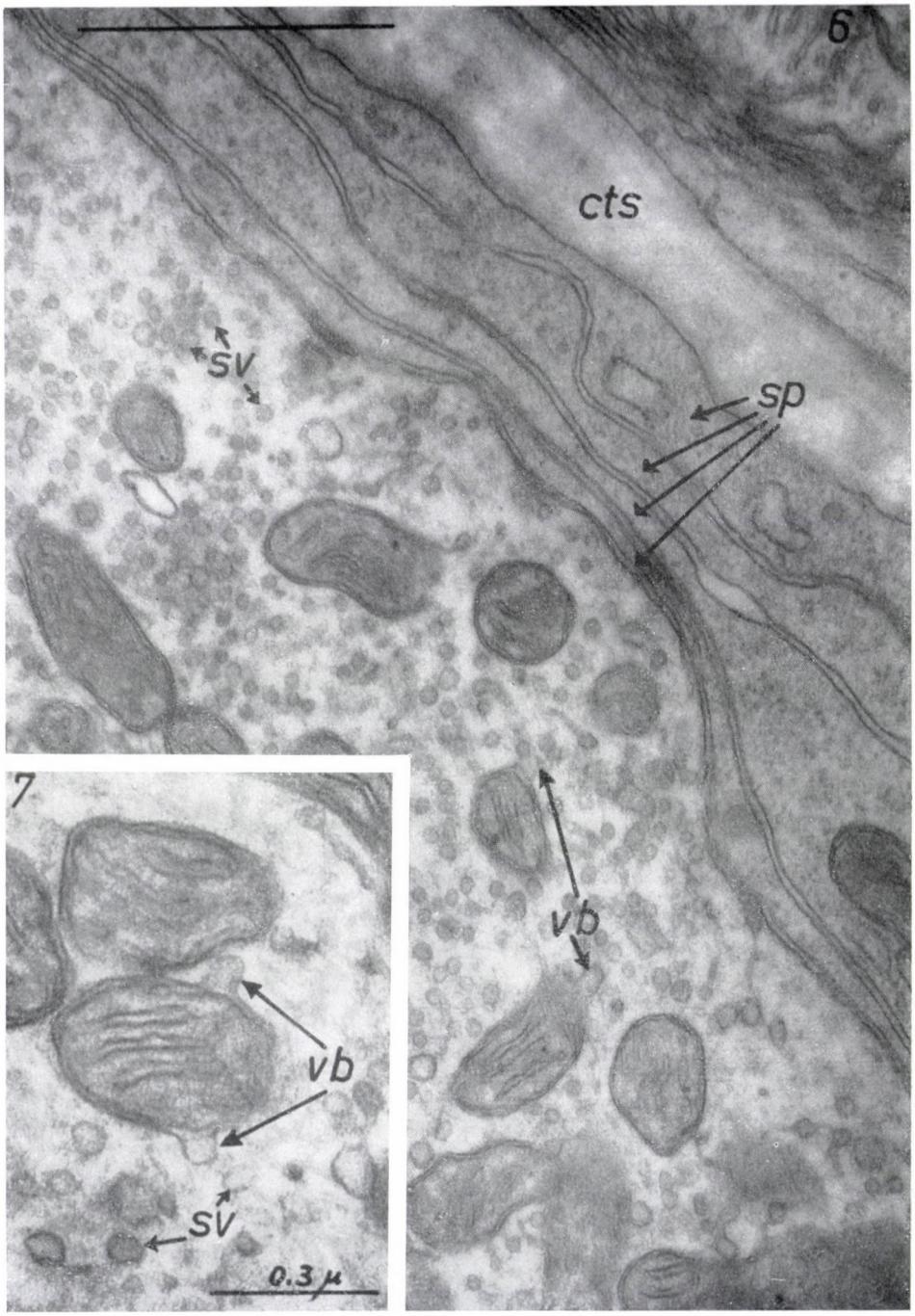
Fig. 1. Low power magnification of axon hillock region of the ganglion cell with synaptic calyx and Schwann cell capsule. Ciliary ganglion of the chick, first day after hatching. *go*— Golgi apparatus of the ganglion cell; *ca* — synaptic calyx; *Sn* — Schwann cell nucleus; *lm* — "loose myelin"; *ao* — axon origin of the ganglion cell; *cts* — connective tissue space. — The area marked with ringed arrows is shown also with higher magnification in Fig. 2



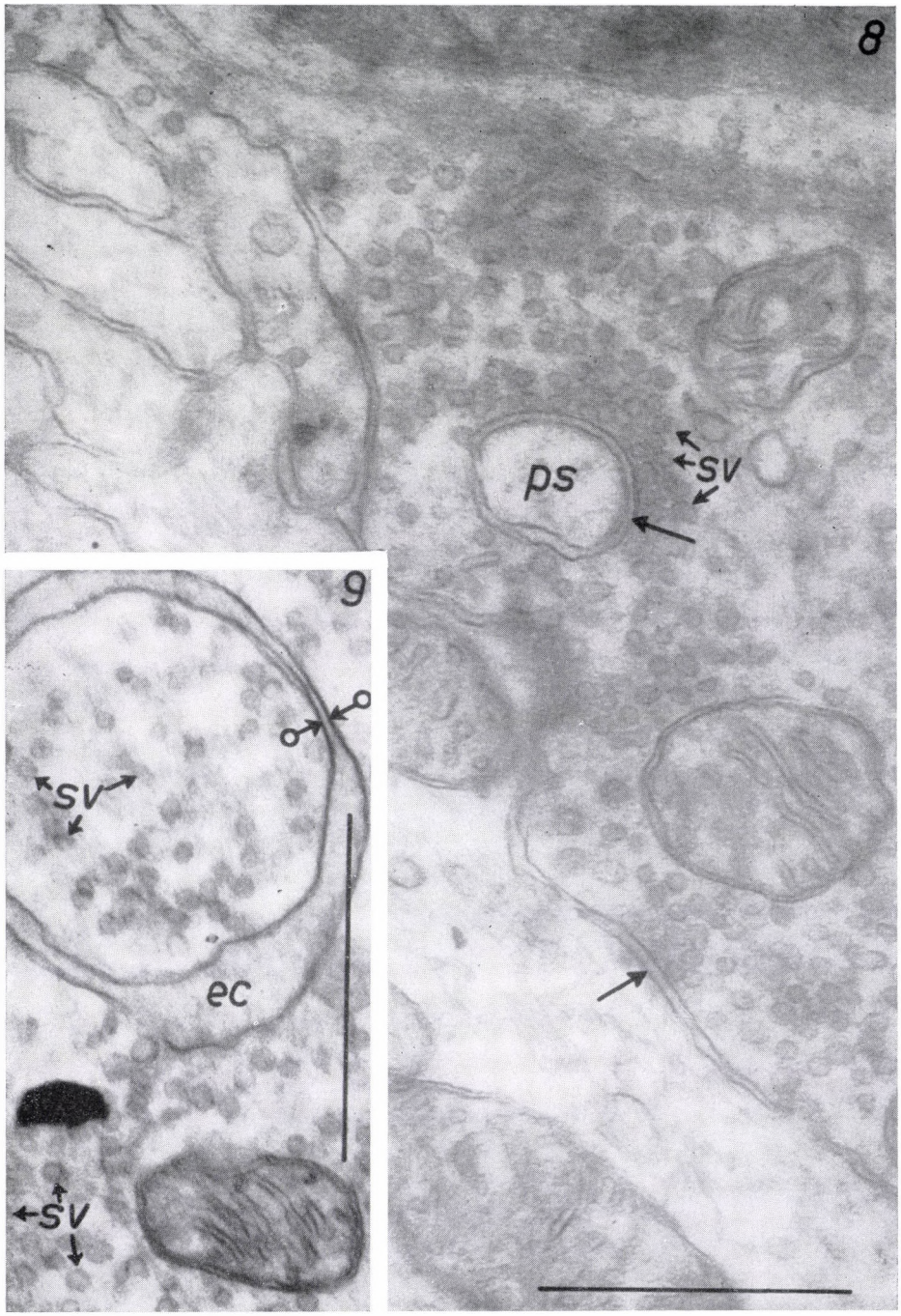
Figs. 2. and *3.* Part of the calyx with higher magnification, from the newly hatched chick, showing only desmosomoid contacts *(dc)* between surfaces of ganglion cell and synaptic calyx. Note the presynaptic vesicles *(sv)* are of various sizes. *Sp* — Schwann cell process.



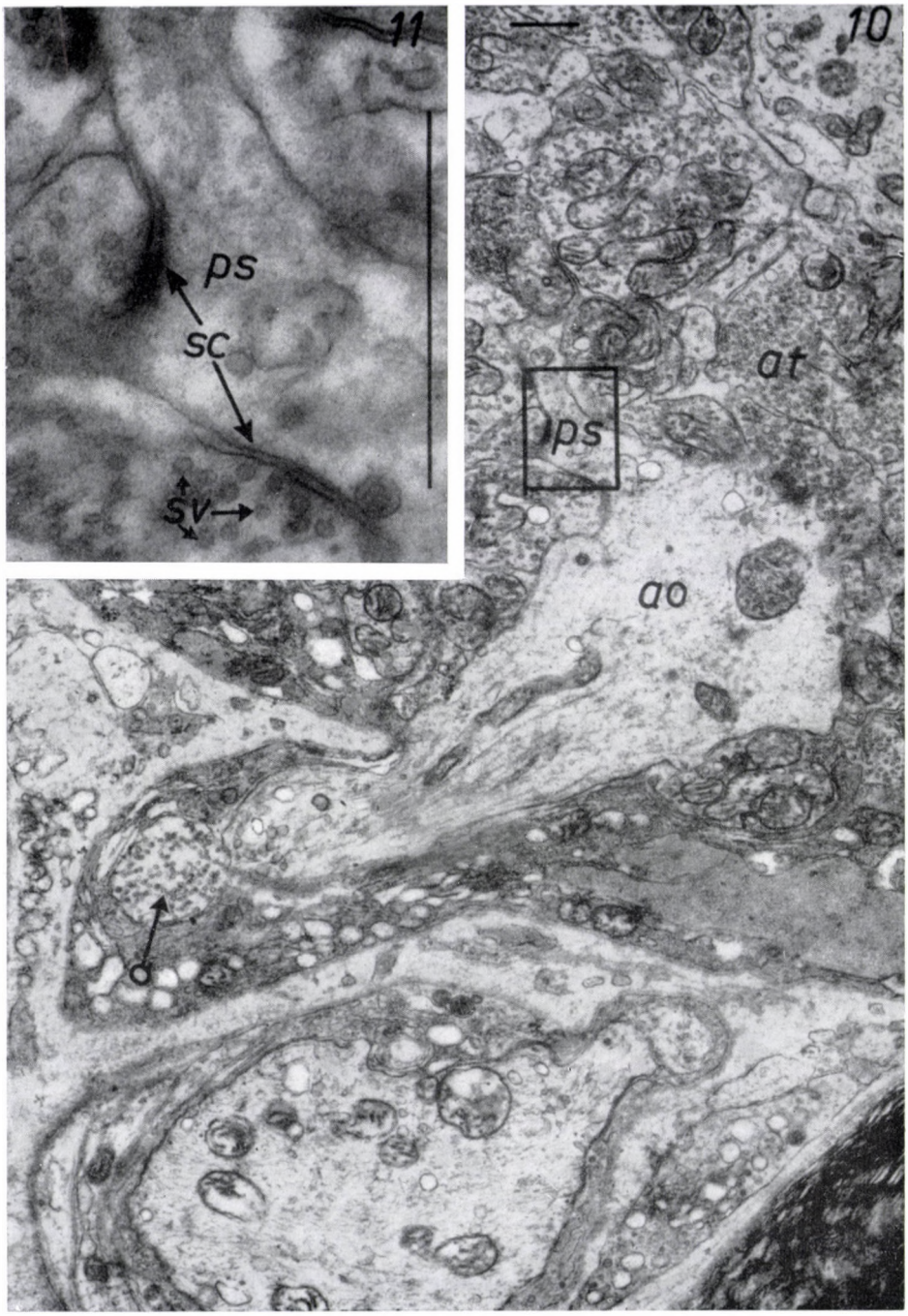
Figs. 4 and *5*. *Fig. 4* is showing part of the solid synaptic calyx *(ca)* from a 1 day chick. The calyx and ganglion cell *(cb)* is isolated from the connective tissue space by a loose myelin-like structure *(lm)*. Initial part of the myelin-sheath of the ganglion cell axon *(ga)* is marked with arrows. In *Fig. 5* an extracellular cleft *(es)* is seen in close connection with desmosomoid contact *(dc)*



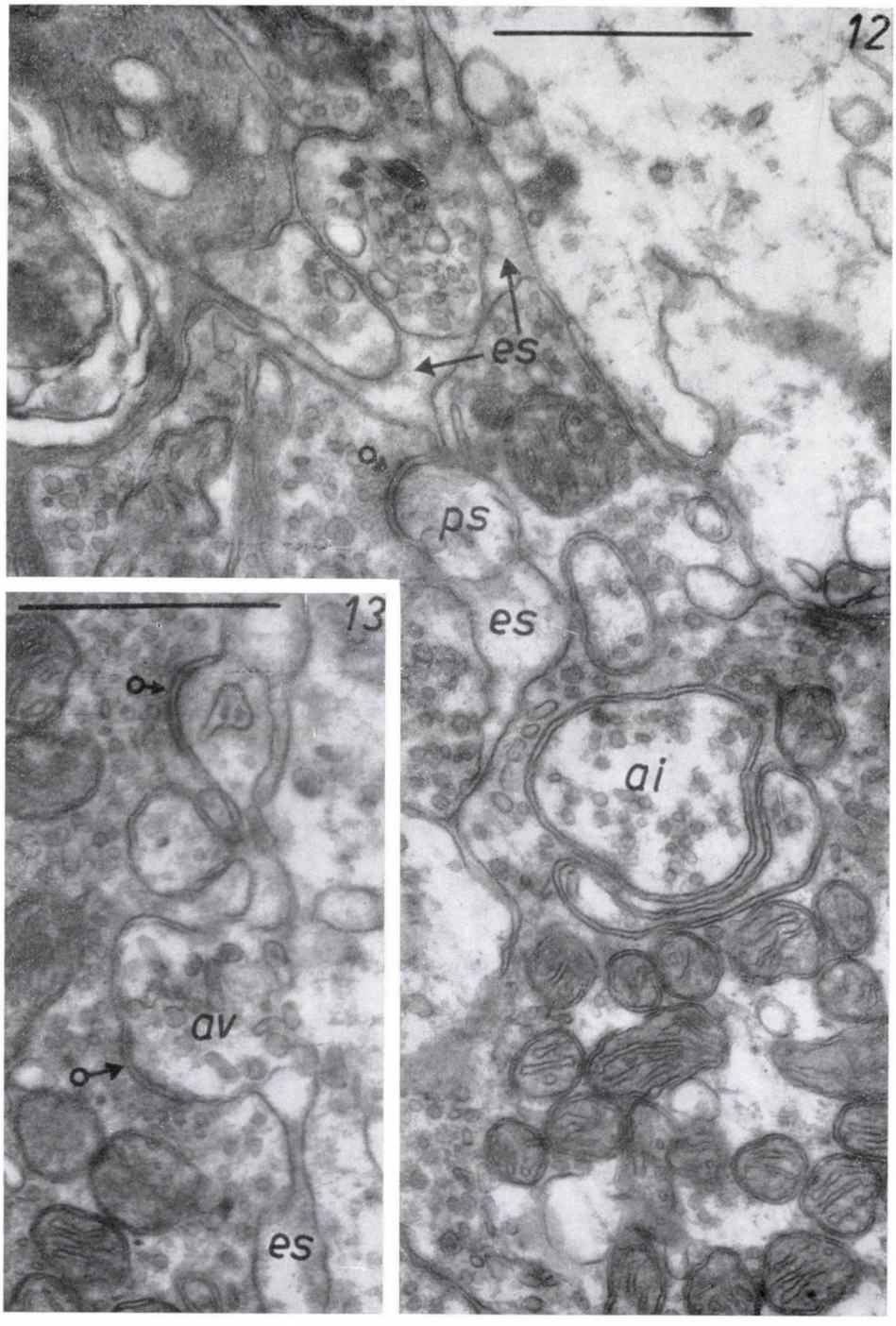
Figs. 6 and *7*. Synaptic vesicles *(sv)* are seen to bulge out *(vb)* from the outer membrane of some mitochondria. "Loose myelin" in *Fig. 6* is shown also with higher power. It consists here of four Schwann cell processes *(sp)* and separates the synaptic calyx from connective tissue space *(cts)*. First day after hatching



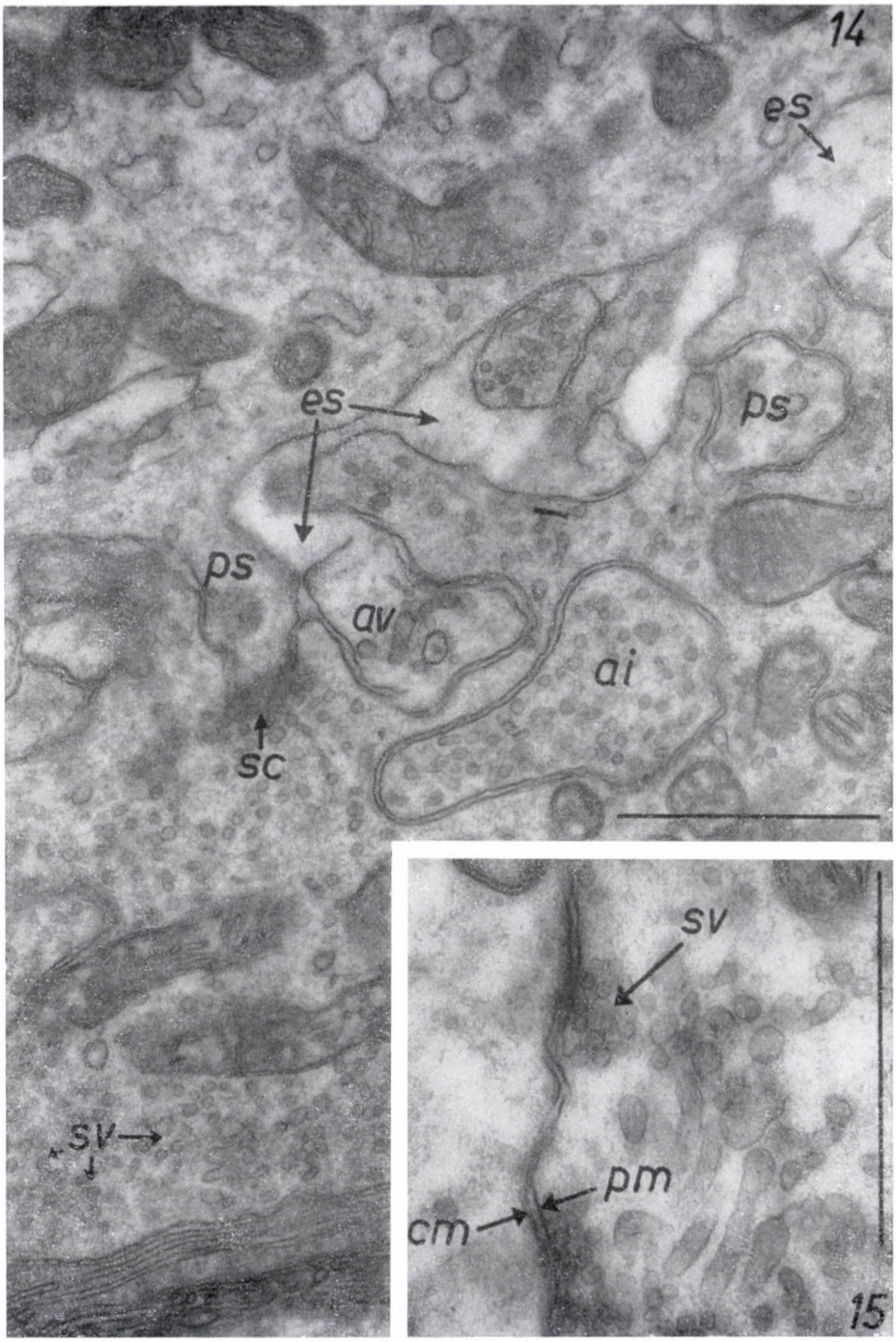
Figs. 8 and *9*. 5th day after hatching. In *Fig. 8* arrows point to synaptic contacts with synaptic vesicle *(sv)* accumulation on the presynaptic site. *ps* — pseudodendrite. In *Fig. 9*. extracellular matrix may be observed between axonic profiles. Ringed arrows show two limiting unit membranes of the neighbouring axonic profiles. That may be interpreted as a result of the subdivision of the originally solid calyx. The clefts produced in that way between axonic profiles will be completely filled by small cell processes only few days later. *sv* — synaptic vesicles



Figs. 10 and *11*. Synaptic calyx covering the axon hillock region and even the initial part *(ao)* of the ganglion cell axon. Note that even leaving the Schwann cell capsule the axon is in contact with small presynaptic axons (ringed arrow). *at* — axon terminal, *ps* — pseudodendrite. Framed part of the calyx with pseudodendrite of the initial part of the axon is shown with higher magnification in *Fig. 11*. It has true synaptic contacts *(sc)* with presynaptic axon branches. 7th day after hatching



Figs. 12 and *13*. On the 7th day after hatching the subdivision of the originally solid calyx is in an advanced stage with development of pseudodendrites *(ps)* and also axonic self-invaginations *(ai)*. Synaptic membranes (ringed arrows) are more osmiophilic than before and aposynaptic vesicles *(av)* are observed on the subsynaptic site. Extracellular space *(es)* between pre- and postsynaptic elements in the vicinity of synaptic contacts is also seen



Figs. 14 and *15*. Pseudodendrites *(ps)* in synaptic contact *(sc)*with the axon terminal from the 9 day chick. *av* — aposynaptic vesicles; *ai* — axon invagination; *es* — extracellular space; *sv* — synaptic vesicles. *Fig. 15* from a 10 day chick showing presynaptic *(pm)* and postsynaptic *(cm)* membrane thickenings with synaptic vesicle *(sv)* accumulation opposite to the presynaptic membrane

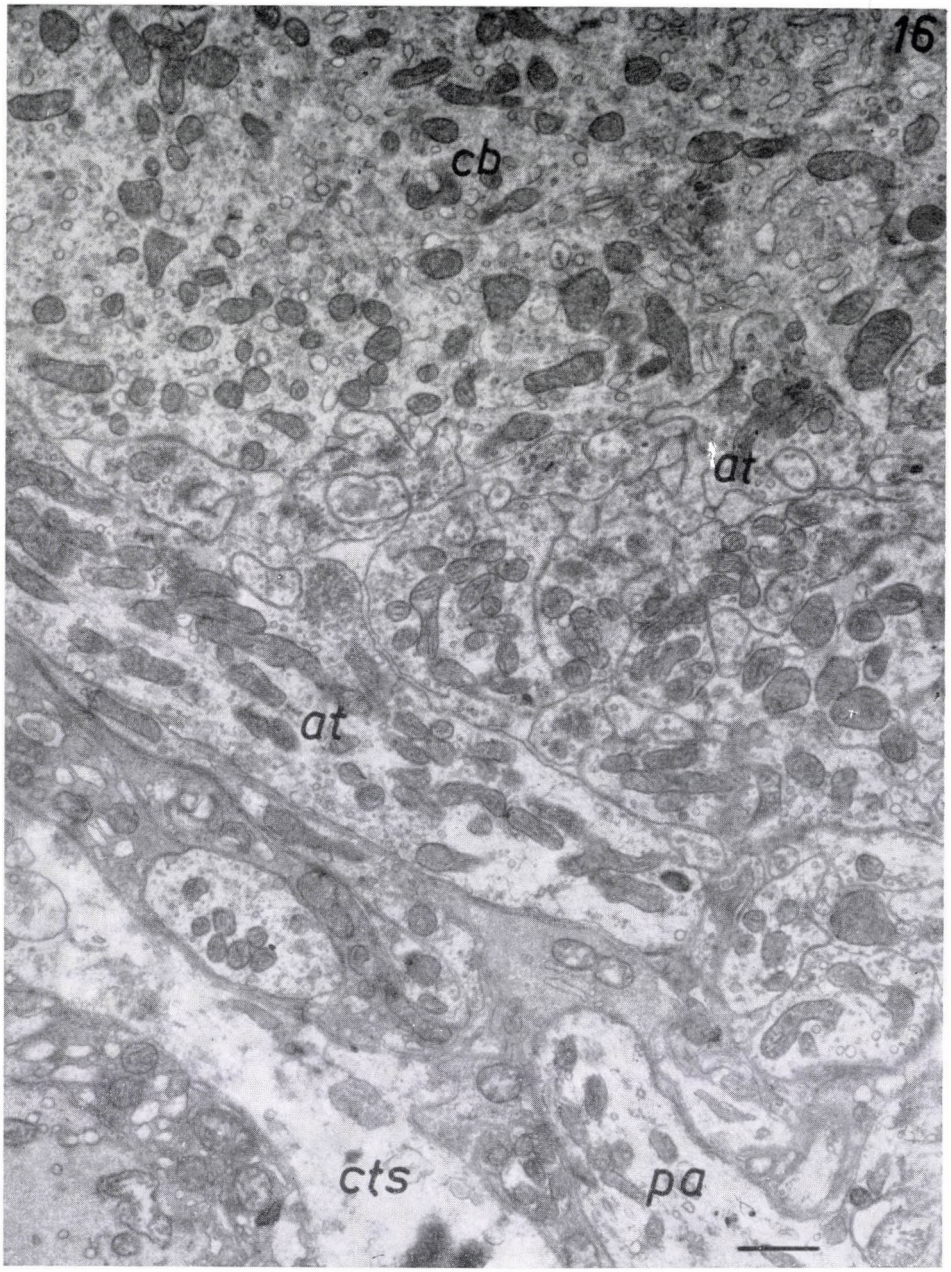


Fig. 16. Figure of the synaptic calyx in the 13 day chick ganglion. See subdivision of the preteyminal axon *(pa)* to several individual axon terminals *(at)* and compare with the solid calyx from the one day chick (Figs. 1 and 4). *cts* — connective tissue space; *cb* — ganglion cell body

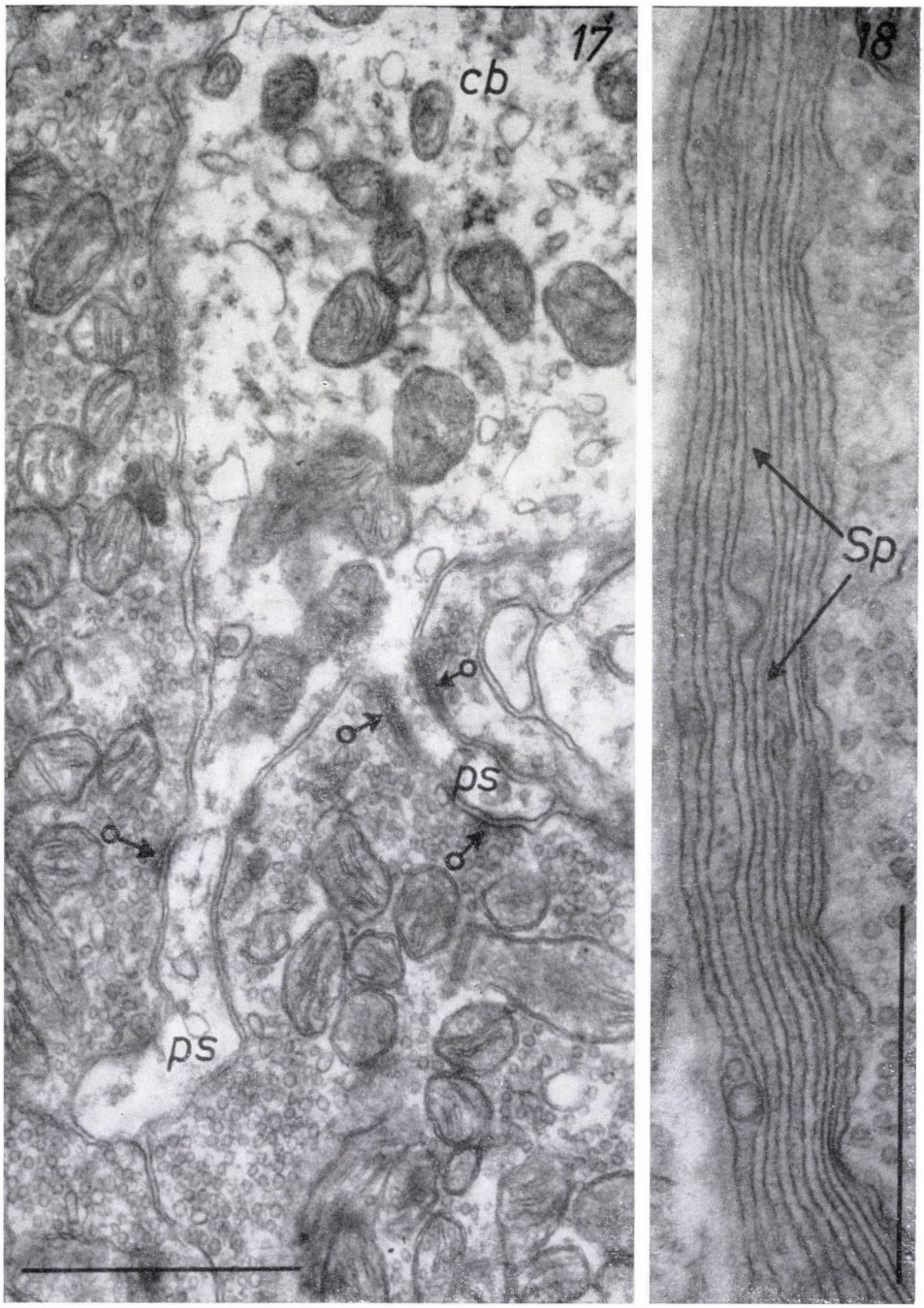
The development of the specific (active) synaptic contact regions is also a remarkable process. In the newly hatched chick there are already some thickened regions of the contact surfaces between solid calyx and ganglion cell body (Figs. 2, 3 and 5) showing some osmiophilia in their neighbourhood, clear accumulations of synaptic vesicles, however, are usually lacking. Thus these contacts have still the character of simple desmosomes. Accumulation of synaptic vesicles begins around the 5th day (Fig. 8) especially opposite the regions of the outgrowth of pseudodendritic processes. The postsynaptic plasma appears first to be empty and the postsynaptic membrane only slightly thickened. On the 7th day the synaptic cleft in the specialized regions of synaptic vesicle accumulation becomes clearly widened (Figs. 12, 13 and 14), the postsynaptic membrane thickened and osmiophilic, and a most remarkable accumulation of relatively large (800-1500 Å diam.) subsynaptic vesicles occurs. Later the subsynaptic plasma region looses somewhat from its characteristic vesicular apparatus (Fig. 15). In the adult or near adult stage the majority of the synapses between the pseudodendrites and the cup-shaped presynaptic labyrinth are typical polarized synapses of Gray's [6] 2nd type. Some axo-somatic membrane thickenings still occur (Fig. 19), but they appear to be symmetric attachments without definite structural polarization. Therefore they may probably be classified as desmosomoid contacts. Similar synaptic contacts occur also between axonal profiles (Fig. 19), as already mentioned by SZENTÁGOTHAI [14]. As they are contacts between the different branches of the same preganglionic fibre, it would be difficult to attach to them any significance beyond purely desmosome nature.

The number of mitochondria and synaptic vesicles shows a marked increase with advancing age. In the younger chicks the synaptic vesicles are much less uniform in size (300—1000Å diameter) than usually seen in adults. Vesicular bulges of the outer membrane of mitochondria and various stages of their separation by alignment (Figs. 6 and 7) might suggest that the synaptic vesicles may be produced here by the mitochondria as already mentioned by DYACHKOVA and associates [1].

Discussion

The early postnatal development of the ciliary ganglion synapse shows a dramatic transformation of the calyciform synapse in a few days' time. In connexion with this spectacular change attention may be focussed on the following three problems:

(1) The observed changes are very quickly increasing the contact surface between presynaptic terminal and ciliary ganglion cell by a factor of about ten, probably even much more. It is interesting to correlate this change with



Figs. 17 and *18. Fig. 17*: synaptic contacts (ringed arrows) between pseudodendrites *(ps)* and axon terminals in the adult ciliary ganglia. In Fig. 18 "loose-myelin" made of five thin Schwann processes *(Sp)* is shown from a 10 day chick. The processes are thinner than in the young ganglia. (Compare with Figs. 4 and 6)

Fig. 19. Three types of specialized contacts from the adult. *ac* — axosomatic, desmosomoid contact; *aa* — axo-axonic desmosomoid contact; *sc* — synaptic contact between axon terminal and pseudodendrite *(ps)*, *mv* — multivesicular body in the pseudodendrite, *cb* — ganglion cell body

the functional change observed by MARTIN and PILAR [11] of an initially mixed chemical and electric transmission mechanism into a purely electrical one during almost exactly the same period. Since, according to ECCLES [3], effective chemical transmission requires a low resistance from the synaptic cleft into the interstitial space, it could well be imagined that, although the synaptic cleft does not change appreciably in width, the tremendous increase of the contact surface would provide a situation more favourable for electric transmission. It could be argued, of course, that by the cleavage of the synaptic calyx into a labyrinth of individual axon branches arborizing in brush-like manner clefts would be opened in sufficient amount for currents to flow towards the outside, which would be more favourable for chemical transmission. It must, however, be kept in mind that only few of the clefts reach the outside of the cup and that even then the whole cleft system is sealed from the outer space by the strong loose myelin system. Additionally the whole labyrinth of the synaptic cup is nothing else but an extremely extended contact between the same two cellular membranes lying always closely parallel each other. The relatively large extracellular cisterns that occur in the initial stages of synaptic cup transformation might be favourable for chemical transmission and their disappearance with advancing age would well agree with the findings of MARTIN and PILAR [11].

(2) It is also interesting to correlate the functional changes with the structural differentiations in the regions of specific contact. In the newly hatched chick there are mostly no accumulations of synaptic vesicles in the regions of adjacent thickened membranes. Thus the thickened regions of the synaptic membranes are structurally non polarized connexions of the desmosome type. Therefore, according to current concepts on the functional significance of synaptic ultrastructural differentitations they should be unsuitable for chemical transmission. Strikingly, however, the synapse of the newly hatched chick has an at least partly chemical transmission which seems to turn into an electric one just in the period during which the most beautiful synaptic vesicle accumulations, i.e. the generally acknowledged submicroscopic apparatus for the release of a chemical transmitter develop. Also the structurally clearly polarized (asymmetric) character of the synapse, which clearly increases during the critical period, is difficult to reconcile with the growing bidirectionality of the synapse as described by MARTIN and PILAR [10, 11]. The lower effectiveness of the backward transmission, on the other hand, would fit quite well with the structural facts. There is no anatomical reason for the assumption of fibres passing through the ciliary ganglion, which, of course, would explain all these discrepancies.

(3) The excessive amounts of acetylcholinesterase found by SZENTÁGOTHAI and co-workers [15] in the bird ciliary ganglion and said to be localized predominantly in the presynaptic endings has also to be taken into conside-

ration. In the newly hatched chick acetylcholinesterase activity is so weak that it is hardly detectable with the usual histochemical methods. It seems very likely, therefore, that it develops parallel with the characteristic synaptic differentiations. This again does not very well harmonize with the general concepts of a predominantly electric transmission.

This analysis is leading up to the conclusion that the correlation between the structural changes occurring in ciliary ganglion synapses during the first few weeks after hatching, and the functional changes found during the same period by MARTIN and PILAR [11] is by no means satisfactory. At least not so in the framework of current concepts on the functional significance of ultrastructural features of synapses.

Acknowledgements

The authors wish to thank Mr. A. KARLOV and Mr. V. KOZLOV for their technical assistance in electron microscopy.

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ENTWICKLUNGSDIFFERENZIERUNG DER SYNAPSEN DES GANGLION CILIARE BEIM HUHN: ELEKTRONMIKROSKOPISCHE UNTERSUCHUNG

Die in den charakteristischen kelchförmigen Synapsen des Ganglion ciliare des Huhnes in den ersten Tagen nach der Ausbrütung zustandekommenden strukturellen Veränderungen wurden elektronmikroskopisch untersucht. In dem frisch ausgebrüteten Huhn ist die synaptische Endigung der präganglionären Fasern eine solide Calyx. Die Unterteilung der Calyx in individuelle Axon-Endigungen beginnt unmittelbar nach dem Herausschlüpfen aus dem Ei. Die Zerspaltung des synaptischen Kelches kommt durch die extracellulären Spalten zustande. die von der Kontaktoberfläche aus sekundär zwischen den Zellkörper und den präsynaptischen Kelch dringen. Manche der Spalten sind von kleinen irregulären Fortsätzen — den sogenannten »Pseudodendriten« — des Zellkörpers des ursprünglichen axonalen Segmentes erfüllt. Desmosomoide Kontakte zwischen den prä- und postsynaptischen Membranen — die jedoch in der spezifischen Ansammlung präsynaptischer Vesikel fehlen — sind in den ersten Tagen zu beobachten. Die Umwandlung dieser strukturell »nicht polarisierten« Kontakte in echte, mit spezifischer Akkumulation der synaptischen Vesikeln einhergehende synaptische Verbindungen kommt hauptsächlich am 5.—14. Tag nach der Ausbrütung zustande. Diese Spezialisation der Kontakte geht von der Axon-Hügel-Region aus und setzt sich in der Peripherie der Calyx fort. Die ganze synaptische Anordnung, die präsynaptische Calyx und den postsynaptischen Nervenzellkörper inbegriffen, ist durch eine von dünnen Fortsätzen der Schwannschen Zellen gebildete »lockere Myelin-Struktur« umhüllt und von der Umgebung isoliert. Eine ansehnliche Menge der zwischen den prä- und postsynaptischen Elementen bestehenden extracellulären Räume ist hauptsächlich bei jungen Ganglien zu finden. Die Quantität dieser extracellulären Räume verringert sich im fortschreitenden Alter in charakteristischer Weise. Im Lichte von an simultanen Veränderungen von Impulsübertragungen gemachten neueren Beobachtungen wird die mögliche funktionelle Bedeutung dieser in dem in Entwicklung begriffenen Ganglion ciliare zustandekommenden strukturellen Veränderungen besprochen.

ЭЛЕКТРОННОМИКРОСКОПИЧЕСКОЕ ИЗУЧЕНИЕ ОНТОГЕНЕЗА СИНАПСОВ В РЕСНИЧНОМ ГАНГЛИИ ЦЫПЛЕНКА

Изучалась ультраструктура синапсов в ganglion ciliare цыплят, начиная с 1-го по 13-й день после выклева, а также у взрослых птиц.

Изменения в онтогенезе касались формирования общей контактной поверхности преганглионарного волокна и ганглиозной клетки, синаптических контактов, структурных элементов пре- и постсинаптических участков и, наконец, оболочек из шванновских клеток.

Контактная поверхность пре- и постсинаптических элементов в этот период увеличивается за счет образования «псевдодендритов» на поверхности клеточных тел, а также выростов на поверхности аксонных окончаний и уменьшения экстрацеллюлярных пространств. Увеличение контактной поверхности сопровождается увеличением числа синапсов.

В процессе формирования отдельных элементов синапсов в онтогенезе вначале происходит уплотнение участков контактирующих мембран, которые на ранних стадиях развития имеют вид десмосом. В дальнейшем формируются типичные синаптические пузырьки, которые скапливаются у пресинаптических мембран. В образовании синаптических пузырьков принимают участие метахондрии. Плотность синаптических мембран повышается, синаптическая щель расширяется. В постсинаптической цитоплазме появляются характерные для этих участков пузырьки и трубочки. К 12—14 дням развития синапсы приобретают типичный поляризованный вид.

Эта специализация синаптических контактов начинается с области аксон-холлока и позднее переходит на периферию калликса.

Отростки шванновских клеток, образующие оболочку вокруг каждой синаптической единицы, в процессе развития увеличиваются в числе, истончаются, плотно прилегают друг к другу и на поздних стадиях развития они напоминают по внешнему виду истинный миэлин. Однако, по характеру своего образования эти оболочки отличаются от миэлина.

Обсуждается возможно функциональное значение структурных элементов синапсов ganglion ciliare в свете последних электрофизиологических наблюдений.

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Acta Biol. Hung., **15** (2), 231—236 (1964)

UPTAKE OF PHOSPHORUS BY ISOLATED DAUCUS TISSUE

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(Received June 29, 1964)

Synopsis

The uptake of P^{32} by tissue cultures of *Daucus carota* L. has been investigated in the function of incubation time. It has been found that after an initial rapid (passive) uptake the rate of penetration of P^{32} becomes steady. The rate of penetration into the interior of the tissue depended on the amount of total phosphorus absorbed. During the incubation time about 25% of the labelled phosphorus added to the cultures penetrated the tissues. The rate of uptake could be enhanced by shaking (increase in uptake by 6—40%). The meristematic regions accumulated phosphorus. P^{32} translocation due to evaporation of water from the surface of the cultures was not observed.

Introduction

A number of authors have dealt with the uptake of substances by various plant organs particularly since the advent of radioactive isotopes [4, 5, 12, 14, 15]. Comparatively little attention was devoted to the ion uptake of and transport in isolated tissue cultures although tissue cultures offer an excellent material for studies of this kind [6, 9, 11].

It has been found that P^{32} accumulates in the meristematic zones and in developing vegetative centres (e.g. the site of root formation) of the oriented tissue tobacco stem [13]. The same was found to be true for *Sequoia* tissues [2, 3]. An accumulation of P^{32} was observed in the cambium of isolated carrot root segments [7]. In other experiments the translocation of phosphorus in carrot was also observed, however no evidence was obtained as to whether the translocation is due to the polarity of tissue (shoot → root direction) or a simple accumulation of P^{32} in the main centres of proliferation is involved [8].

In view of the problem outlined above concerning the causes and circumstances of ion uptake and translocation in tissue cultures experiments were started to obtain a deeper insight into the kinetics of absorption and the nature of transport. A detail of the experiment is described in the present paper.

Materials and methods

Isolated tissues of carrot *(Daucus carota* L.) were used throughout the experiments. The tissue was kept in culture for several years and subjected to subculturing. The material used for the experiments was in a phase of intensive growth, 3 to 4 weeks after subculturing.

The tissue can be regarded as a "callus" in which the elements of vascular tissues start to appear (cells with various types of cell wall thickening).

The isolated tissue fragments were grown on a solid White medium [16] containing salts and the "A—Z micro-elements" of HOAGLAND. Iron was administered as ferricitrate. The medium was supplemented with cysteine (10 mg/l), thiamin (1 mg/l), and β-indoleacetic acid (0.07 mg/l). As a carbon source saccharose (30 g/l) was applied. The pH of the nutrient was 5—5.2 at the time of isolation.

The isolates were grown in flasks, each containing 50 ml nutrient, at room temperature. The cultures were illuminated by diffuse light for 12 hours every day. The colour of the tissue was green at places in contact with the nutrient. Other parts were greenish-yellow.

Cylinders (11 mm in diameter) were punched from the tissues with a cork borer perpendicularly to the surface of the nutrient. The tissue cylinders were cut into disks of various thickness, using an instrument with parallel cutting surfaces. The disks were placed into a liquid nutrient (50 ml/flask/disk) of the same composition as described above (agar omitted) and 0,2 μC/ml $KH_2P^{32}O_4$ was added. The disks immersed into the solution were incubated for various periods at room temperature in diffuse light. The cultures were shaken with a Dubnoff precision shaking incubator at a rate of 100 revolutions/minute.

The tissues were washed twice with distilled water and dried in air steam. The disks were sliced to equal thickness, placed on plates and their radioactivity was immediately counted by an end-window GM tube (thickness of end-window: 2.5 mg/cm^2). After 24 hours the radioactivity was measured again both in the original position of the disks and in reversed position. The data presented are average values.

The author thanks for the original tissue material which has been obtained from EVA PETRU, Institute of Experimental Botany, Czechoslovak Academy of Sciences, Praha. Thanks are also due to ESTER NÁDASSY for technical assistance.

Results

Results of investigations on the rate of P^{32} uptake as a function of incubation time are shown in Table 1. 6 mm $\times$ 11 mm $\varnothing$ excised cylinders were cut at the end of the incubation period into 2 mm thick disks. The radioactivity of the disks was measured. It was assumed that the values obtained are indicative of the rate of uptake and of the speed of translocation. The number of total impulses and the amount of phosphorus calculated from these figures are given. It appears that the uptake of P^{32} by carrot tissue cylinders during 5, 30 or 60 minutes of incubation corresponds to a ratio of 1:2.5:4. Thus, the rate of penetration is not linear, but after an initial rapid uptake the absorption becomes lower. This is clearly indicated by the percentage values of uptake (26.3; 58.6; 100%). The various 2 mm thick disks exhibit different phosphorus uptake. This is partly explained by their different position in the original tissue cylinder. However, the disks deriving from the two ends of the cylinder also contain different amount of absorbed P^{32}. This difference is particularly evident with 60 minutes incubation. Some of the differences might be explained by the morphological changes accompanying sectioning. Thus the surface of the tissue cylinder is approximately 397 mm^2, whereas that of the single disks 322 mm^2. However, differences in the physiological state of the tissues at the two ends of the cylinder (e. g. meristematic tissues or not) can also contribute to the differences in ion absorption. The phosphorus uptake of the inner disks is much lower than that of the outer ones. Only 22—24 per cent of total phos-

Table 1

Phosphorus uptake of tissue disks of isolated carrot (Daucus carota L.) tissue

Weight of tissue in mg	Incubation time in minutes	Orientation of the disk	Treatment	P-uptake γ/disk		P-uptake γ/cylinder	P-uptake %	P-uptake of the inner disk in % of total uptake
519 ±11.6	60	outer	shaked	5.99	±0.014	18.72	100	22.0
		inner		4.12	±0.026			
		outer		8.61	±0.056			
528 ±13.7		outer	not shaked	4.28	±0.075	13.83	73.8	24.8
		inner		3.44	±0.085			
		outer		6.11	±0.147			
519 ±11.6	30	outer	shaked	4.41	±0.033	10.98	58.6	22.4
		inner		2.47	±0.086			
		outer		4.10	±0.098			
528 ±13.7		outer	not shaked	3.48	±0.109	9.78	52.3	26.4
		inner		2.59	±0.017			
		outer		3.71	±0.054			
519 ±11.6	5	outer	shaked	1.64	±0.049	4.43	23.6	23.4
		inner		1.04	±0.033			
		outer		1.75	±0.020			
528 ±13.7		outer	not shaked	1.30	±0.014	3.14	16.7	26.4
		inner		0.83	±0.026			
		outer		1.01	±0.030			

phorus uptake is recovered in the inner tissues. Thus the time-dependence of penetration and the phenomenon of translocation is evident.

The rate of uptake depended also on the treatment of the cultures (shaked or not). Shaking increased the P^{32} uptake of tissue cylinders by 25% as compared to the unshaken control (incubation for 5 or 60 minutes). As to the P^{32} uptake of the inner disk shaking had no marked influence. The percentage stimulation values are as follows: 60 min. incubation 2.8% (22.0 → 24.8), 30 min. incubation 4% (22.4 → 25.4), 5 min. incubation 3% (23.4 → 26.4). This clearly indicates that shaking hardly effects the P^{32} uptake of tissue portions not being directly in contact with the medium.

To study the transport of phosphorus in isolated tissues the following experiment was carried out. The disks of 315 mg weight and 322 mm^2 surface were directly placed into the liquid medium. The disks were incubated for 60 or 30 minutes. After incubation the radioactivity of the disks was measured. The disks were then placed in open Petri dishes and the radioactivity was measured after 24 hours at both sides of the disks. These experi-

ments were aimed at the elucidation of the effect of transpiration on the translocation of phosphorus.

The results summarized in Table 2 indicate that the total amount of P^{32} absorbed is in no direct relation either with the weight of the tissue or with its surface. However, evidently the surface plays a major role in determining the rate of absorption. The effect of incubation time on the uptake of P^{32} was similar to that observed in the experiment described above. About 31—52% of the total phosphorus uptake takes place during the first 30 minutes in case of 2 mm thick disks. With 6 mm thick disks the corresponding figures are 52 – 58%. Shaking affected the P^{32} uptake of the thinner disks more than that of the thicker ones. The effect of shaking was more pronounced with longer incubation (Table 2). A 20% increase was experienced with 30 min. incubation in contrast to the 40% increase found with 60 min. incubation.

No effect of transpiration on the movement of phosphorus was observed (Table 2).

Discussion

The experiments were aimed at the elucidation of the rate and circumstances of phosphorus absorption (eventually of P^{32} translocation) by isolated *Daucus carota* tissues. Under the experimental conditions applied an initial high rate of absorption was observed which was followed by a lower rate of P^{32} uptake. It is quite probable that the first phase which lasts for a few minutes corresponds to the passive phase of ion uptake whereas the second one is presumably an active (metabolic) process [10]. About 24% of the total phosphorus uptake takes place during the initial period. More than the half of total absorption (52—59%) is accomplished during the second period of half an hour. The characteristics of the uptake process are similar with the unshaken variant (17% and 53%) although the absolute values are somewhat lower. The lower rate of uptake by unshaken disks is explained by the lower P^{32} concentration at the surface of the tissue.

The rate of penetration was clearly indicated by the differences of the amount of P^{32} present in the various parts of the tissue cylinder incubated with P^{32}. Generally, one fourth of the total phosphorus taken up by the cylinder was found in the middle (inner) disk (22—26%). This figure was independent of the incubation time. It follows from this observation that the rate of penetration is linear. Differences between the rate of uptake of the two outer disks might be explained partly by differences in weight and surface due to sampling difficulties. The size of surface is particularly important from the point of view of ion absorption. However, physiological differences between the two ends of the tissue cylinder should also be taken into account. A similar, preferential accumulation of P^{32} was observed by SCHMITZ [13] in the meristematic

Table 2

Phosphorus uptake of isolated tissue disks of carrot (Daucus carota L.)

Weight of tissue in mg	Treatment	Incubation time in minutes	P-uptake		P-uptake*	
			γ	%	γ	%
318 ±3.39	Shaked	60	20.13 ±0.46	100	17.54 ±0.95	83.16
		30	10.59 ±0.36	52.60	10.21 ±0.77	50.72
	Not shaked	60	11.91 ±0.70	59.16	11.95 ±0.74	59.36
		30	6.40 ±0.77	31.79	5.93 ±1.09	29.50

* Measurement repeated after 24 hours on reversed disks.

regions of tobacco callus tissue. The radioautographic investigations of BALL [2, 3] in *Sequoia* tissues point in the same direction. HELLER [7, 8] also observed a marked ion accumulation in the cambial region of carrot callus tissue, and a polarized transport and accumulation of P^{32} in oriented tissues. However, he was unable to differentiate the translocation due to true polarity from that induced by the proliferating tissue centres. Thus it might be assumed that the more intense uptake and accumulation is elicited by more than one factor. In our material the differences in uptake might also reflect a complex situation. Although cytological investigations were not carried out in the present study, previous observations indicated that the two ends of the tissue cylinder might contain cells of different physiological state. In the callus tissue meristematic centres are easily formed and the tissue cylinder might be composed of meristematic and more differentiated regions. These meristematic regions are distributed in the callus tissue at random. This explains the failure to obtain differences in P^{32} uptake between the two ends of the tissue cylinder in some cases. Therefore, true polarity of the tissue could not be established.

Further experiments were conducted to throw some light on the cause of P^{32} translocation within the tissue cylinder. According to the widespread opinion the main factor regulating P^{32} translocation in plant organs is the concentration of nutrients and transpiration. The effect of these factors is obvious in case of contact uptake as soon as after 5 minutes [1]. In our experiment (Table 2) the movement of phosphorus due to transpiration could not be demonstrated.

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PHOSPHOR-AUFNAHME VON ISOLIERTEN DAUCUS-GEWEBEN

Die Phosphor-Aufnahme von isolierten *Daucus carota* L. Gewebekulturen wurde in Abhängigkeit von der Inkubationszeit mit Hilfe von P^{32} untersucht. Es konnte festgestellt werden, daß nach einer einleitenden, schnellen (passiven) Phase die Aufnahme von P^{32} langsamer und gleichmäßig erfolgt. Die Geschwindigkeit der Aufnahme ist von der Gesamtmenge des absorbierten Phosphors abhängig. Ungefähr 25% der Gesamtaufnahme erfolgt im Laufe der einleitenden, schnellen Aufnahmeperiode. Die P^{32}-Aufnahme konnte durch Schütteln der Kulturen um 6—40% erhöht werden. Eine P^{32}-Akkumulation durch meristematische Gewebe konnte festgestellt werden. Dem gegenüber hat Verf. eine transpirationsbedingte Erhöhung der P^{32}-Aufnahme nicht beobachtet.

ПРИЕМ ФОСФОРА ИЗОЛИРОВАННОЙ ТКАНЕЙ DAUCUS CAROTA

Автор при помощи P^{32} изучал поглощение фосфора культурой изолированной ткани моркови (Daucus carota L.) в зависимости от времени инкубации. На основании экспериментальных данных установил, что после начального быстрого (пассивного) поглощения, поступление фосфора в кусочки ткани становится равномерным. Ритм поступления внутрь ткани обнаруживает связь с общим количеством поглощенного фосфора, примерно 25%-ов от общего количества поступает во время инкубации. Встряхивание повышает поглощение на 6—40%. Наблюдалось на меристематических областей повышенное накопление фосфора, но возникающую вследствие испарения воды тканей транслокацию фосфора автор не обнаружил.

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Acta Biol. Hung., **15** (2), 237—249 (1964)

THE EFFECT OF TSH ON THE ULTRASTRUCTURE OF EMBRYONIC CHICK THYROID

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Synopsis

The thyroids of 8-, 11-, and 14-day-old intact and decapitated chick embryos were examined with the electron microscope. In intact embryos the ergastoplasm of the thyroid cells was found to be increased in quantity and changed in quality from the 11th day while this phenomenon does not occur after decapitation at midbrain level. Adding TSH or transplanting hypophysis of the same age to decapitated embryos the above described phenomenon could be established. The earliest effect of TSH was observed 1—1 1/2 hour after a single injection. The mechanism of follicular development is also demonstrated and discussed.

Introduction

As the radioautographic experiments of HÁMORI, MESS and SZÉKELY [5] carried out in 1960 have shown, the fetal chick thyroid begins to store I^{131} on the 11th embryonic day. On the base of this finding authors stated that the fetal chick hypophysis starts to produce TSH on the 11th day and this results in the I^{131}-storage of the thyroid. However, in spite of this fundamental functional change, only slight parallel morphological alterations could be observed on the thyroid epithelial cells, i.e. the cells of the accumulating gland were light-microscopically nearly identical to the not accumulating ones. This observation and a further investigation of MESS and STRAZNICKY [7] suggested the present work. These authors emphasized that the mentioned morphological identity is only due to the insufficient resolving power of the light microscope. Therefore, this electron microscopical study was planned to answer the following question: are there any ultrastructural changes in the thyroid of decapitated chick embryos compared to the intact ones?

Material and method

The experiments were performed on 8-, 11-, and 14-day-old chick embryos. In the first experimental group the embryos were decapitated at midbrain level on the 2nd day of incubation as described elsewhere [10]. The second group served as control. TSH-preparation "Ambinon" (Organon) was administered to decapitated embryos in 2 USP mU quantity 1/2-,1-, 2-, 3-, 6- and 12 hours preceding the sacrification of the embryos, dropped on the chorioallantoid membrane. To an other group of decapitated embryos hypophysis taken from 7 day old embryos were transplanted under the abdominal skin between the right wing and leg on the 6th day of incubation, before 8 days of sacrification, respectively. The thyroids were removed under dissecting microscope, fixed in 1 per cent OsO_4 solution buffered at pH 7.5 for 1 hour, embedded in Araldite [6]. Ultrathin sections were cut with LKB-Ultrotome, floated on distilled water, picked up on copper grids without any film coating, stained with the lead nitrate method and observed with Zeiss (Jena) EL-MI II. and Tesla B 242 electron microscopes.

Results

Ultrastructurally the thyroid of the 8-day-old intact embryo (Fig. 1) shows a rather poor cytoplasmatic pattern. Characteristic large mitochondria and Golgi-zones are visible, but the endoplasmic reticular system is only poorly developed. A few of rough surfaced vesicles are scarcely distributed in the cytoplasm. In some cases between neighbouring cells cavities with numerous microvilli are formed. These cavities correspond to the early intercellularly developed (Fig. 8) follicles in contrast to those which develop intracellularly as it will be described later.

The thyroids of the 8-day-old decapitated embryos do not differ from the thyroids of intact animals of the same age.

In the thyroids of the intact 11-day-old embryos the significant change compared to the previous stage is the appearance of an increased lamellar-type ergastoplasm (Fig. 2a). Though in the 8-day-old embryos some rough surface vesicles are already visible, they resemble neither in quantity nor in quality to the ergastoplasm of the 11-day-old fetal thyroid. Mitochondria, Golgi-tubules and vesicles, as well as randomly distributed secretory granules are also present.

The thyroids of 14-day-old chick embryos show an increase of the above mentioned structures, especially of the ergastoplasm (Fig. 3). Intracellular colloid droplets are formed which are in many cases fusing with each other and later with the colloid droplets of neigbouring cells, forming this way regular follicles (Figs. 7*a* and *b*). This represents the formerly mentioned intracellular way of follicular development. Capillary sinusoids with endothelial wall are already well developed at this age.

The effect of decapitation is striking if examined on the 11th or 14th day (Figs. 2*b* and 4). The ergastoplasm observed of the thyroid cells of decapitated non treated embryos of this age is nearly totally absent, and the cytoplasmic pattern is very similar to that of the 8-day-old thyroid. However there is one important difference, namely the Golgi-material is developed undisturbedly without any sign of decapitation effect experienced on the ergastoplasm.

TSH-treatment was performed on 13-day-old decapitated embryos. 24-hours after the administration of Ambinon (2 USP mU) the above described characteristic ergastoplasm is well developed and follicles though in initial stage were found (Fig. 6). From the increased amount of ergastoplasm, however, it was supposed that its development requires a shorter period. To determine the time of the earliest manifestation of TSH effect, thyroids were fixed 1/2-, 1-, 2-, 3-, 6- and 12 hours after TSH-administration, and it was found that the process begins at about the first hour with the elongation of the originally round rough surfaced vesicles. This continues until the ergastoplasm

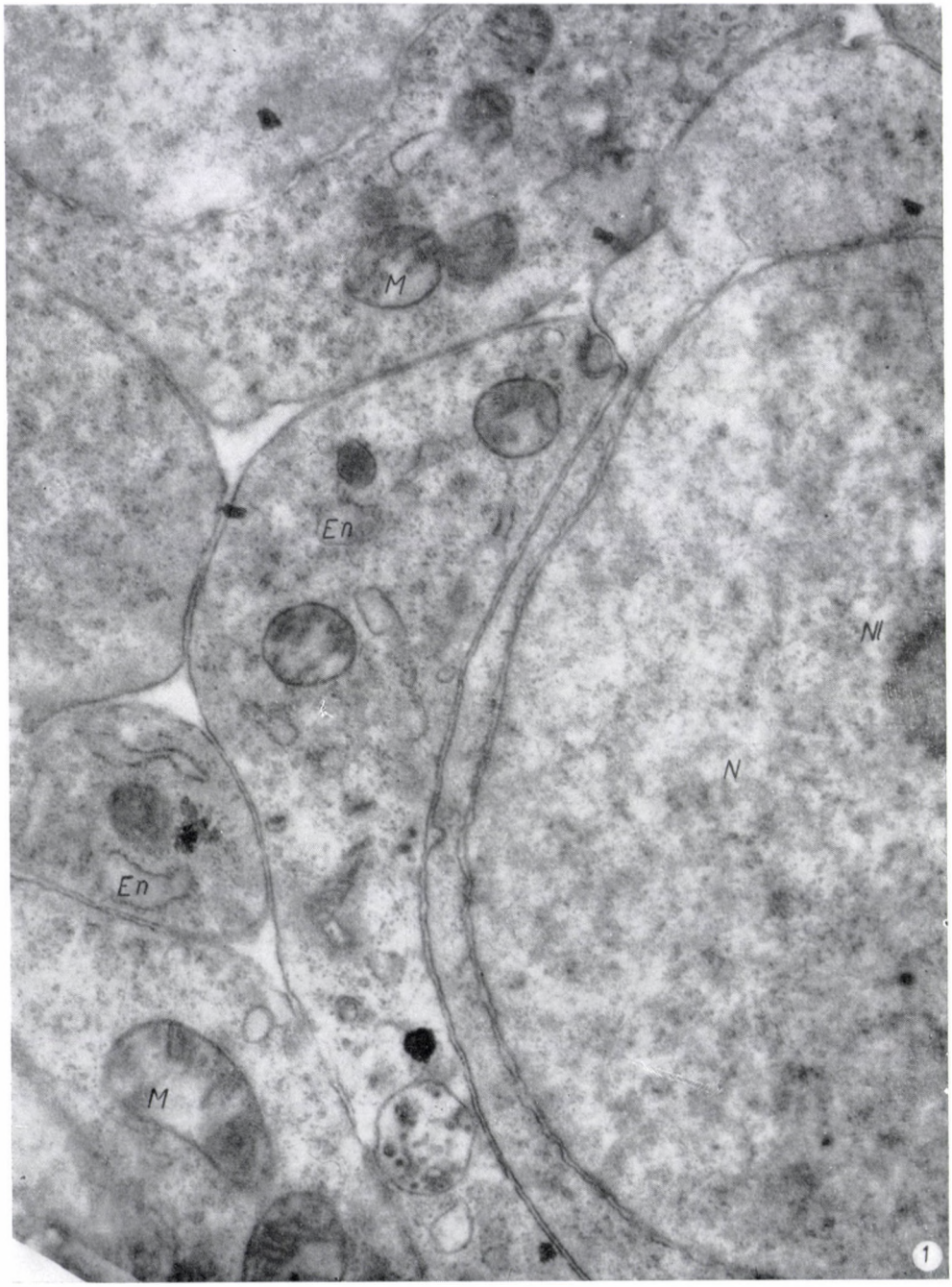


Fig. 1. Thyroid of the 8-day-old chick embryo. The cytoplasmic structures are poorly developed. En—endoplasmic reticulum, M—mitochondria, N—nucleus, Nl—nucleolus, × 21,000

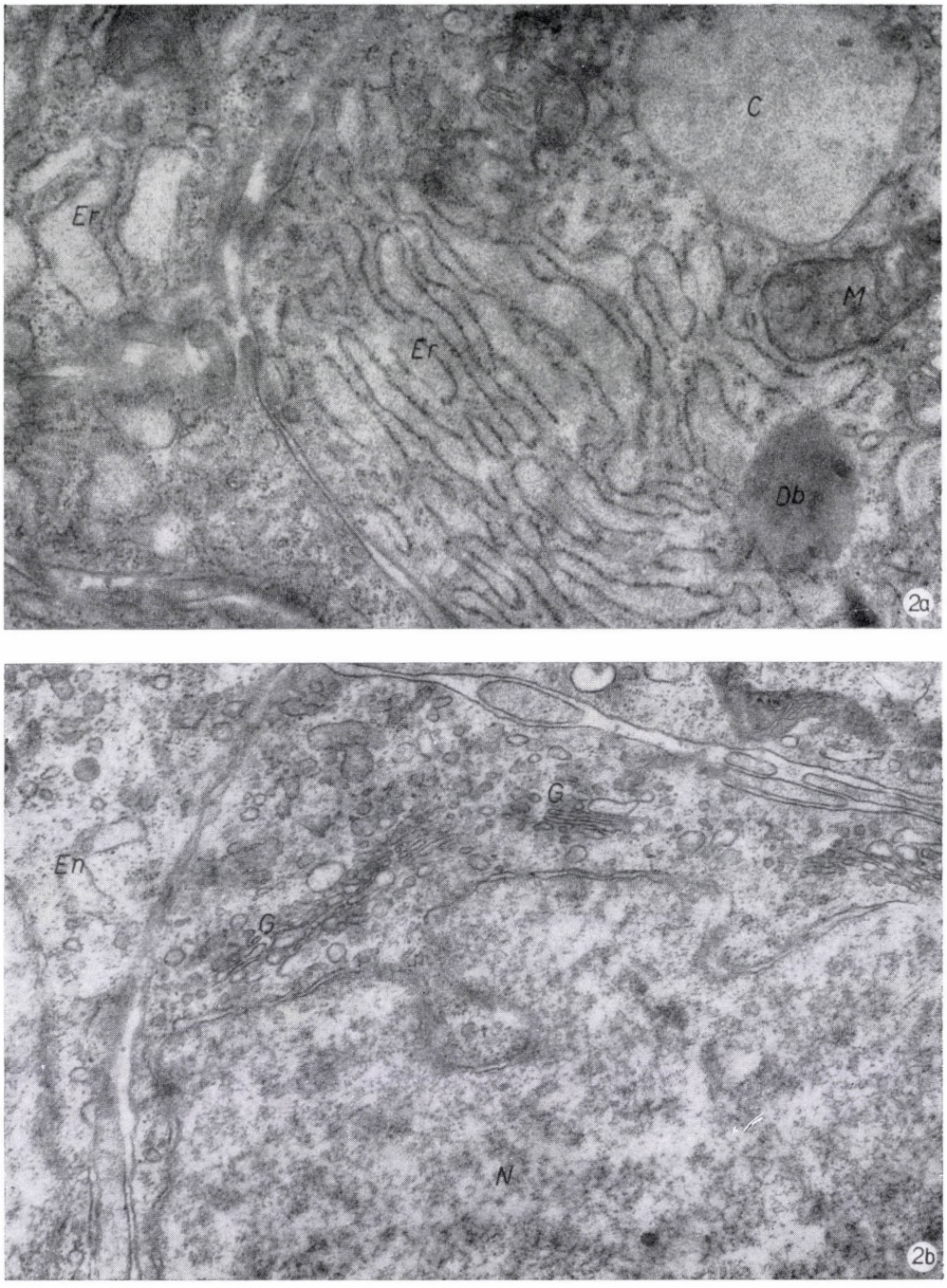


Fig. 2. a — Thyroid of the 11-day-old chick embryo. The ergastoplasm is already well developed ×18,000. *b* — Thyroid of the 11-day-old decapitated chick embryo. The cytoplasm is nearly empty, only the Golgi-material is visible. ×18000 C—colloid, Db—dense body, En—endoplasmic reticulum, G—Golgi-material, M—mitochondria, N—nucleus, Er—ergastoplasm

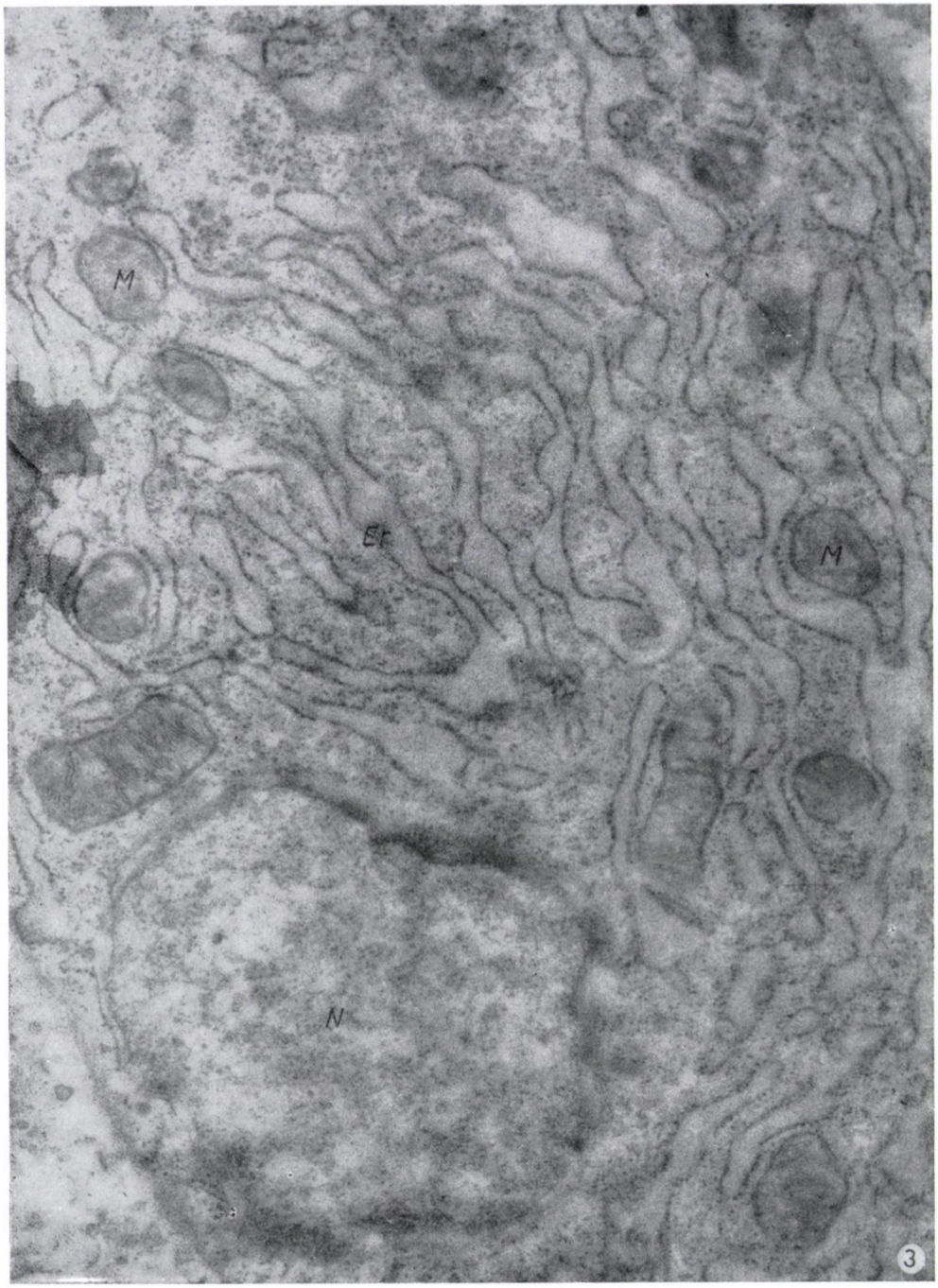


Fig. 3. Thyroid of the 14-day-old chick embryo. Note the increased amount of ergastoplasm. ×21,000. Er—ergastoplasm, M—mitochondria, N—nucleus

receives its described lamellar form at about 6 hours following a single TSH injection (Figs. 5*a* and *b*).

According to radioautographic data [5] it was expected that the embryonic thyroid is already reactive for TSH before the 11th day and this supposition was proved by the fact, that ergastoplasmic proliferation could be provoked already from the 7th day of incubation.

The TSH-treatment of decapitated embryos was occasionally substituted with hypophysis transplantation, to obtain continuous physiological TSH-level. The operation was performed on the 6th day and the fixation of thyroids on the 14th day, the results were nearly identical to those of the TSH-treatment. The only difference is the presence of well developed large follicles, surrounded by numerous cells.

During this process, independently from the way of their genesis, the cisternae of the ergastoplasm are filled with a very finely granulated material showing the same electron density as the intracellular and intrafollicular colloid (Fig. 2*a*). In the surrounding of the follicles some vesicles 500—1000 Å in size are visible and their number is markedly increasing by TSH-administration (Fig. 6).

Discussion

Most of the authors dealing with the ultrastructure and ultrastructura differentiation of the embryonic thyroid [1, 2, 3, 11] state that the rough surfaced endoplasmic reticulum is rapidly increasing from a certain stage of fetal development. This day is in the case of chick embryo the 11th, when a new lamellar type of the endoplasmic reticular system is appearing, termed as ergastoplasm. These findings are supported by the present investigation and the TSH-dependence of this ergastoplasm is proved directly by the decapitation effect and TSH-treatment. Probably the TSH-dependent ergastoplasm has some role in the iodide-transport as well as in the synthesis of the hormone. This assumption might be supported with the fact, that the TSH elicits very quickly — already 2—3 hours after a single injection — the proliferation of a rough surfaced endoplasmic reticulum. The vesicles visible in the surrounding of the follicles might be also involved in the secretory process, evidences in this respect however are still scanty [4, 8, 12]. The precise role of the ergastoplasm in the iodide-transport and the nature of microvesicles is hoped to be explored by experiments in progress in this laboratory with the method of electron microscopic radioautography [9].

Beside the data belonging to the main question of this work other remarkable phenomena were observed, such as the independence of Golgi-material from the thyrotrophic hormone and the different types of follicular development. The latter was reported first by FUJITA and co-workers [3]. In the

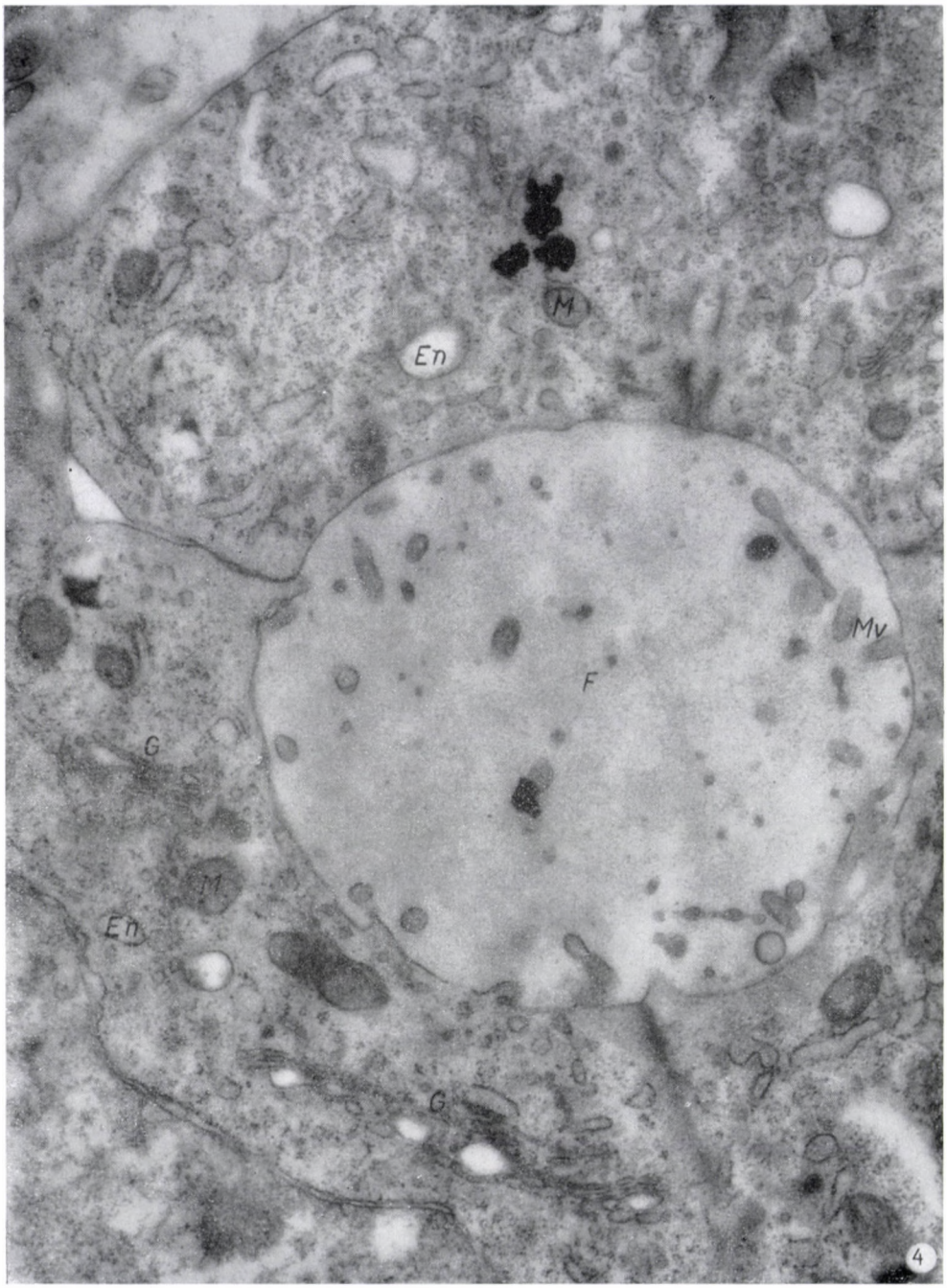
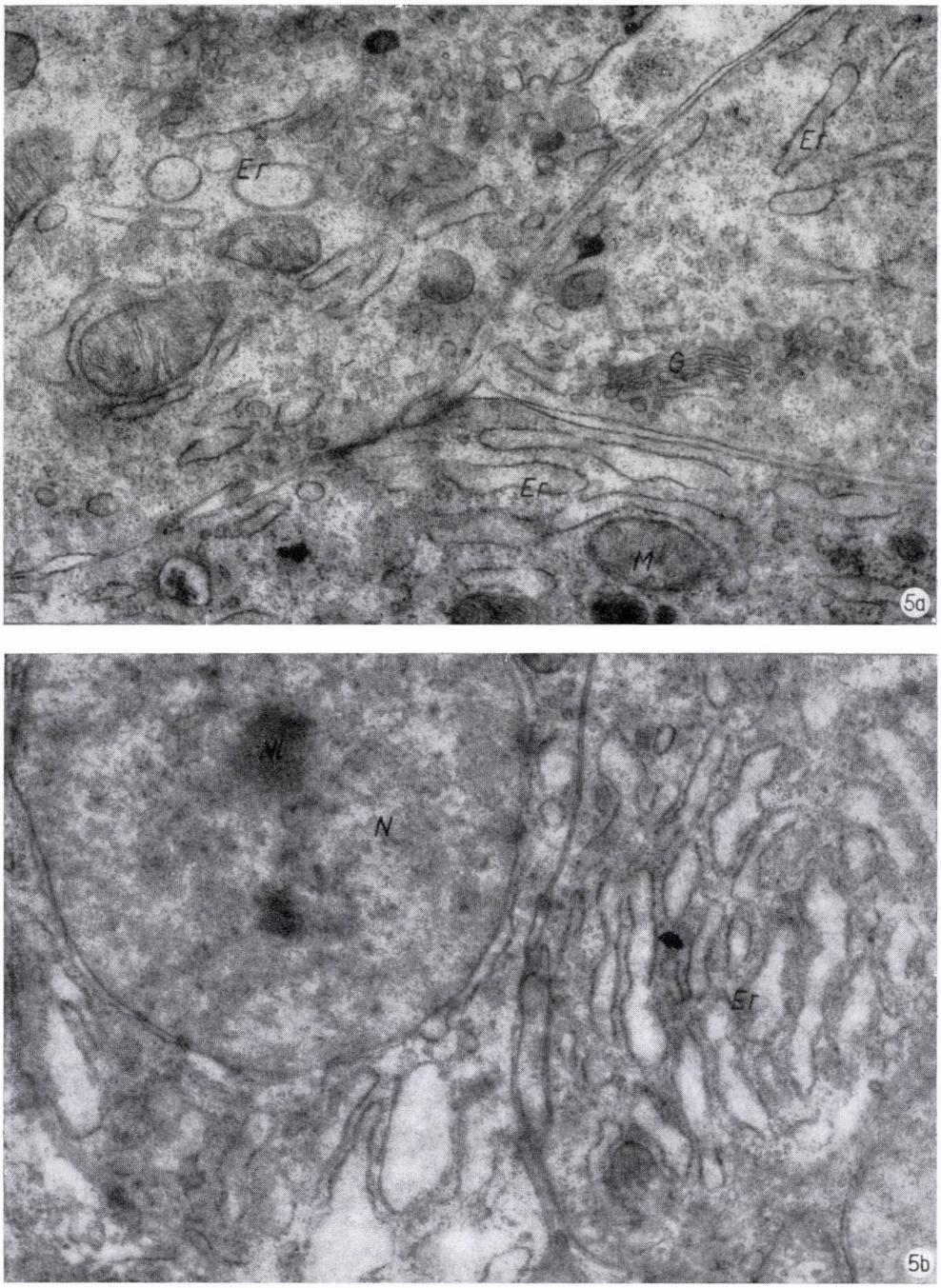


Fig. 4. Thyroid of the 14-day-old decapitated chick embryo. Note the evidence of proliferation of the Golgi-material and the absence of the lamellar-type ergastoplasm. ×21,000. En—endoplasmic reticulum, F—follicle, G—Golgi-material, M—mitochondria, Mv—microvilli



Figs. 5 a and *b*. Thyroid of the 14-day-old decapitated chick embryo 1 and 3 hours after TSH-administration, respectively. Gradual elongation of the vesicular-type of ergastoplasm into a tubular form. × 18,000. En—endoplasmic reticulum, G—Golgi-material, M—Mitochondria, Er—ergastoplasm, N—nucleus, Nl—nucleolus

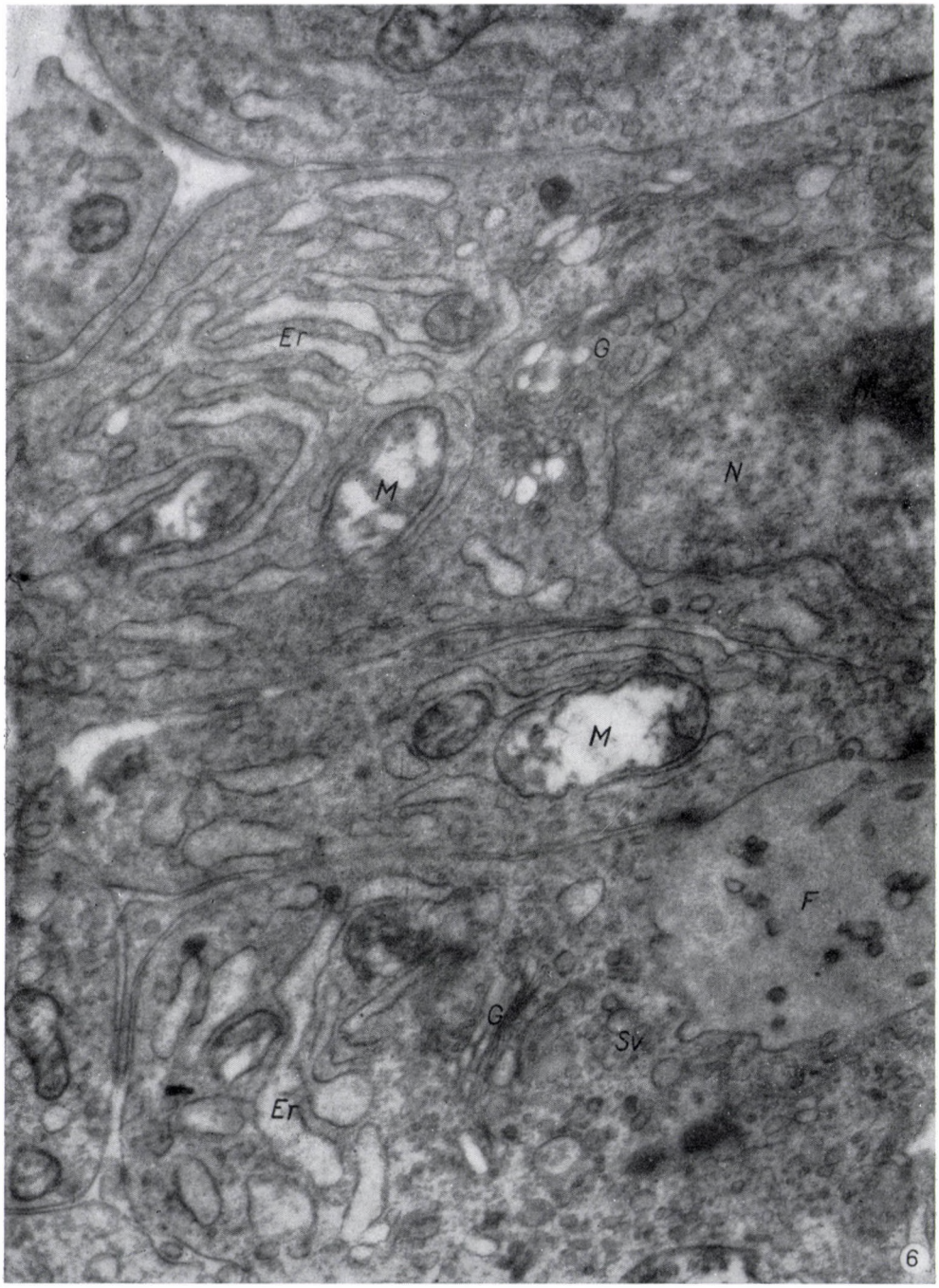


Fig. 6. Thyroid of the 14-day-old decapitated chick embryo 24 hours after TSH -administration. The tubular form ergastoplasmic vesicles differentiated into a lamellar-type of ergastoplasm. ×21,000. Er—ergastoplasm, G—Golgi-material, M—mitochondria, N—nucleus, Nl—nucleolus, Sv—small vesicles

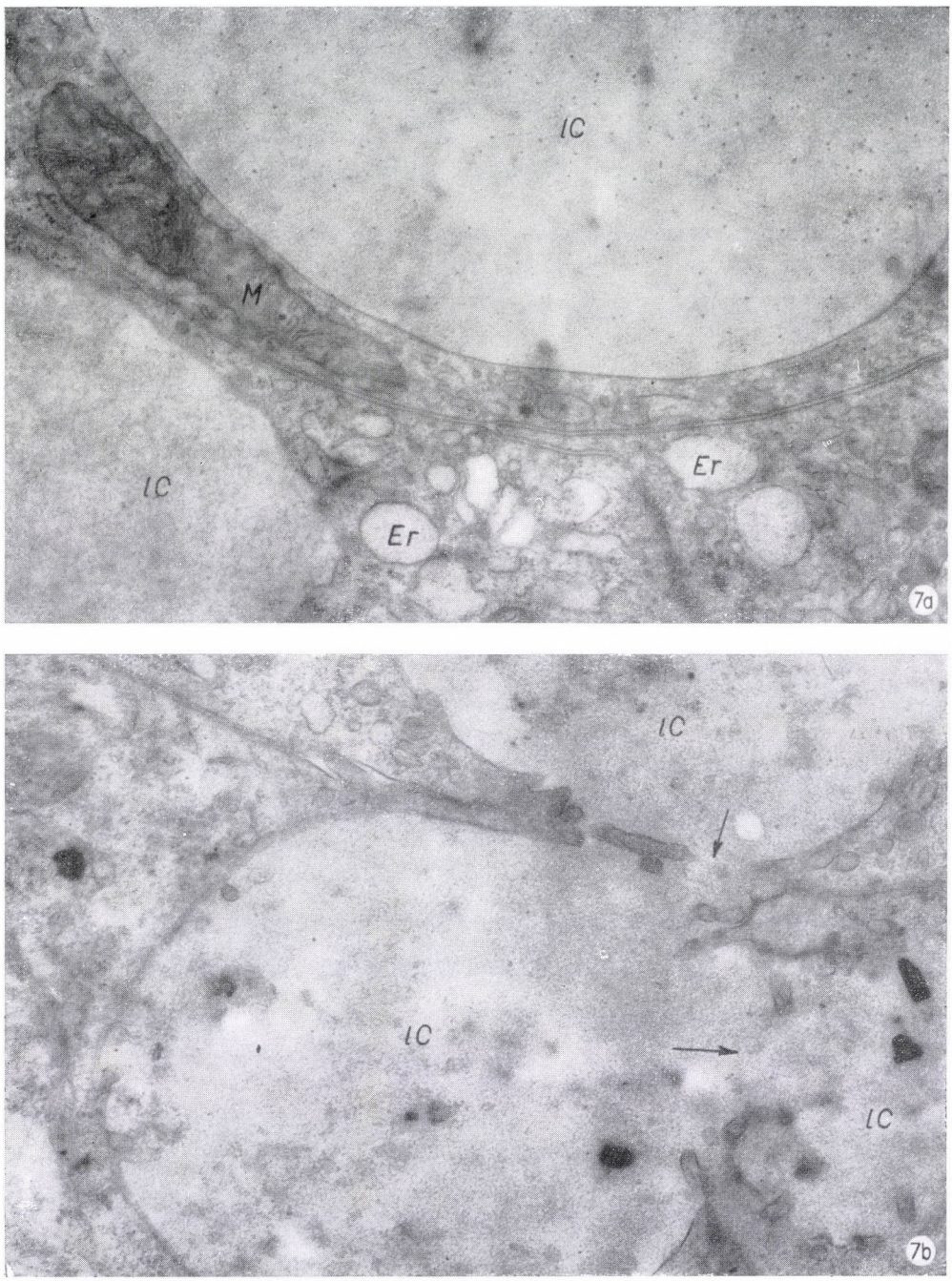


Fig. 7. Intracellular type of follicle development. *a)* The enlarged intracellular colloid droplets are getting near to each other. *b)* The fusion of the neighbouring colloid droplets is indicated with arrows. × 18,000. En—endoplasmic reticulum, IC—intracellular colloid droplets, M—mitochondria, N—nucleus, Nl—nucleolus

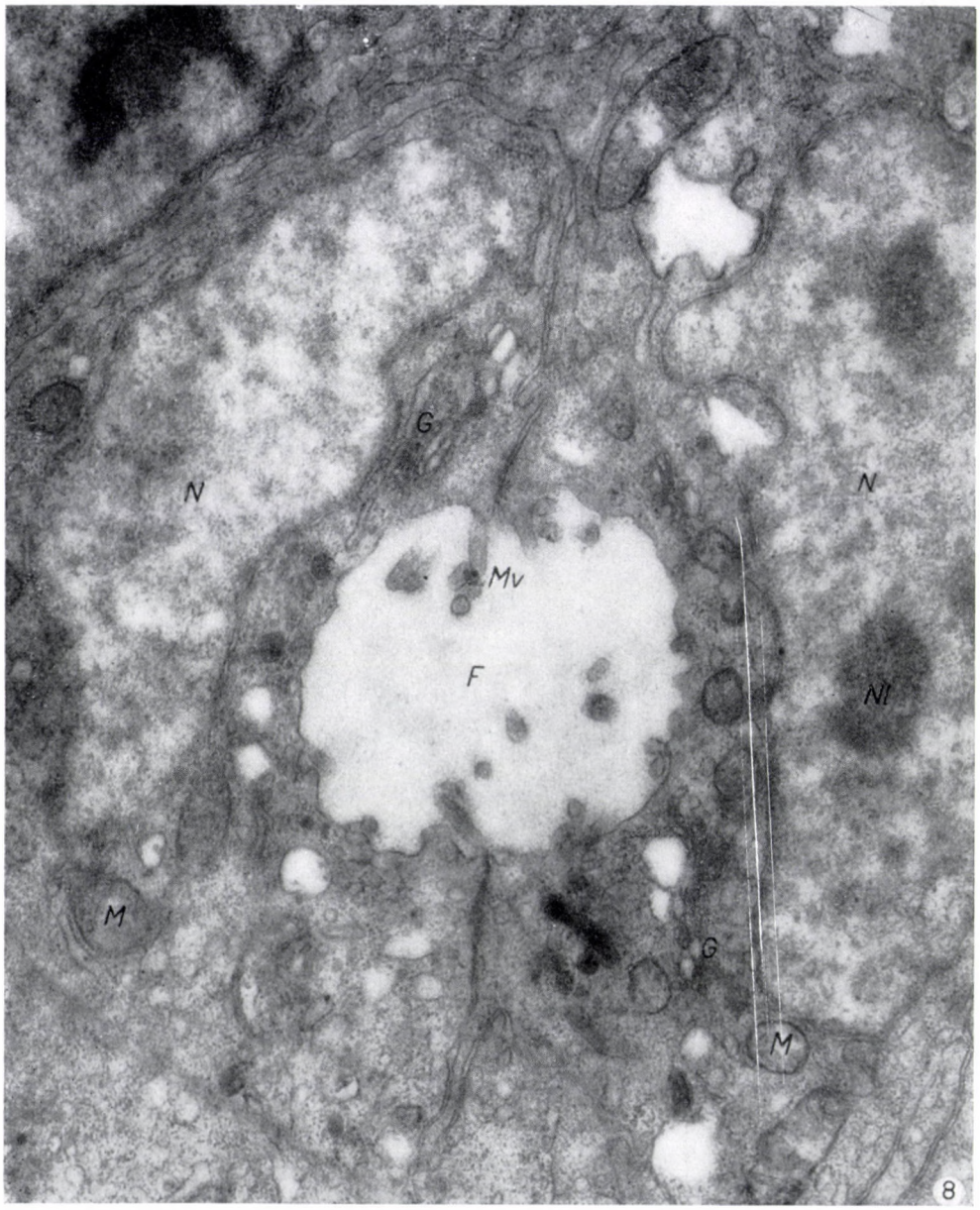


Fig. 8. Intercellular-type of follicle development. Characteristic to the thyroid of younger embryos. ×12,000. F—follicle, G—Golgi-zone, M—mitochondria, Mv—microvilli, N—nucleus, Nl—nucleolus

material presented here the intercellular mechanism was found to be characteristic of the younger embryos, while the follicles starting their development in the relatively older thyroids showed mostly the intracellular type of growth.

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DIE WIRKUNG DES TSH AN FEINSTRUKTUR DER SCHILDDRÜSE DER HÜHNER-EMBRYONEN

Schilddrüsen normaler und dekapitierter Hühnerembryonen wurden am 8-, 11-, 14. Tage der Bebrütung elektronenmikroskopisch untersucht. Die Schilddrüsen der normalen Embryonen zeigten vom 11. Bebrütungstag eine deutliche Vermehrung des Ergastoplasma mit gewissen qualitativen Veränderungen. Dasselbe konnte im Niveau des Mesencephalons dekapitierter Embryonen nicht beobachtet werden. Nach TSH Verabreichung oder nach Transplantation embryonaler Hypophysen-Vorderlappen (aus Embryonen von gleichem Alter) konnten die bei normalen embryonalen Schilddrüsen gefundenen Veränderungen auch festgestellt werden. Die ersten durch die TSH-Injektion verursachten Veränderungen erschienen schon in 1—1 1/2 Stunden nach der Verabreichung des Hormons. Der Mechanismus der Follikel-Entwicklung wird ausführlich behandelt.

ВЛИЯНИЕ ТИРЕО-СТИМУЛИРУЮЩЕГО ГОРМОНА НА УЛЬТРАСТРУКТУРУ ЩИТОВИДНЫХ ЖЕЛЕЗ КУРИНОГО ЭМБРИОНА

Исследовались щитовидные железы с помощью электронного микроскопа у 8-, 11- и 14-дневных нормальных и декапитированных куриных эмбрионов. Эргастоплазма начала увеличиваться в количественном отношении и изменяться качественно после 11 дней, этого явления не наблюдалось при перерезке на уровне промежуточного мозга. При помощи введения тирео-стимулирующего гормона или пересадки гипофиза из такого же возраста животных декапитированным особям можно вызвать выше описанное явление. Самый ранний эффект тирео-стимулирующего гормона наблюдается через час-полтора после единственной инъекции. Также описывается и дискутируется механизм развития фолликулов.

FERENC HAJÓS
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Acta Biol. Hung., **15** (2), 251—256 (1964)

STUDIES OF THE ALKALOID CONTENT IN CERTAIN SPECIES AND HYBRIDS OF NICOTIANA

I. SEPARATION AND IDENTIFICATION OF TOBACCO ALKALOIDS BY THIN-LAYER CHROMATOGRAPHY

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Synopsis

In the rapid qualitative and semi-quantitative determination of the alkaloid content in different tobacco species and their hybrids the thin-layer chromatographic method proved to be useful. In our technique the adsorbent layer was, as usual, silicagel prepared with 0.5N potassium hydroxide. Subsequently the chromatograms were activated at 110° C for 30 minutes. To determine the sensitivity of the method and to estimate the value of the developing reagents four different tobacco alkaloid preparations (nicotine, nornicotine, anabasine and nicotyrine) were used as standard in concentrations from 0.1 to 10.0 μg. The chloroform-ethanol mixture (90 : 10) gave the best separation of the spots. For the detection of spots a spray of 2 per cent aniline in ethanol followed by a treatment of BrCN vapour, proved to be very suitable. It was possible to demonstrate that the spots of alkaloids developed by the different indicator reagents exhibit a characteristic fluorescence in ultraviolet light, and therefore the identification of the alkaloids in the various tobacco samples is very easy. The parallel use of standard alkaloid preparations in known concentration on the same chromatogram provides the possibility of an approximative quantitative estimation of alkaloid quantities in the various tobacco samples.

Introduction

During our investigations on tobacco genetics the alkaloid content of several species and hybrids has been determined. For a rapid qualitative and quantitative analysis the thin-layer chromatography was found the most suitable method which has been successfully applied for separation and detection of various alkaloids [7, 9]. Testing the constituents of different narcotics and toxins in gastric juices and urine MACHATA [5], BÄUMLER and RIPPSTEIN [1], COHIN and DALY [2] separated nicotine, TEICHERT and co-workers [7] nicotine and anabasine, PAPP and SZABÓ [6] detected also nornicotine on thin-layer chromatograms.

None of the above mentioned papers deals with a special and systematic investigation of tobacco alkaloids. The present paper gives a brief summary concerning our experiences in connection with the detection of some tobacco alkaloids, their separation in various solvent mixtures and with different identification methods.

Material and method

18 g of silicagel (E. Merck) was mixed with 50 ml 0.5 N KOH and the slurry applied uniformly at 0.25 mm with a commercial spreading device (Desaga, Heidelberg). The coated plates are air-dried for 15 minutes at room temperature and then heated in a drying oven for 30 minutes at 110° C. Among the different solvent mixtures tested (chloroform—ethanol = = 90 : 10; chloroform—ethanol = 80 : 20; chloroform—methanol = 100 : 20; pure methanol; chloroform—ether = 85 : 15; toluene—methanol—chloroform = 90 : 30 : 10; methanol—n-butanol—benzene—water = 60 : 15 : 10 : 15;ethanol— acetic acid—water = 60 : 30 : 10) the mixture of chloroform—ethanol = 90 : 10 was found best. The walls of the glass tank are covered with filter paper to make an atmosphere saturated with vapour. Chromatograms are developed at room temperature for 30—40 minutes with ascending technique.

At first sensitivity limits were determined with four authentic preparations (1. nicotine, Merck, puriss.; 2. (—)-anabasine, Fluka, purum; 3. β-nicotyrine, Fluka, purum; 4. nornicotine) as usual and the most adequate reagents were chosen for detection of the spots. From the standard alkaloids solved in chloroform the following quantities were applied on the starting points: 0.1; 0.25; 0.50; 1.0; 2.5; 5.0; 7.5; and 10.0 μg (Fig. 1).

For identification the chromatograms were tested in UV-light, then one of the following reagents were used: Dragendorff-reagent, aniline or benzidine and BrCN vapour, isatin-reagent.

In our investigations alkaloids were extracted with the mixture of benzene and chloroform (9 : 1) from ground and sifted tobacco-dust, according to CUNDIFF and MARKUNAS [3] after addition of $Ba(OH)_2$. When the extract contained too much pigment, a great amount of chlorophyll was extracted by 70 per cent ethanol and petroleum ether (1 : 1) mixture. After concentrating in vacuum the rest was put in known quantity of chloroform and this solution was applied on the plates.

Results and discussion

On our chromatograms only nicotyrine spots were fluorescent in UV-light. Even a low quantity of it (0.1 μg) can be seen as a nicely limited, faint pink fluorescent spot. After treating with benzidine and BrCN nicotine shows a beautiful glow red fluorescence, and nornicotine gives a fainter red fluorescent spot. After the detection with aniline and BrCN nicotyrine shows an intense citrine, nicotine a bright blue and nornicotine a faint pink fluorescent spot.

In some cases the fluorescence after detection implies a very serious help in identification. For instance by analysing the alkaloid extract of a tobacco hybrid of the nornicotine type *(N. tabacum* × *N. glutinosa)* we established on the basis of examination in UV-light after chemical treatment that it does not contain nicotine.

Best results were obtained by treatment with aniline and cyanogen bromide. This way 0.1 μg of the used authentic alkaloid samples were to be detected and their identification facilitated by the fact that the spot colours of certain alkaloids differ very explicitly (Table 1).

The detection with 1% benzidine in methanol and BrCN vapour is sensitive enough and its chief advantage is that the colours do not faint rapidly and can be readily established though all the spots are shaded reddish.

Dragendorff reagent, modified by MUNIER, shows not at all the same sensibility against the different tobacco alkaloids (Table 1). It is most sensitive to nicotine; 0.1 μg of nicotine, 0.25 μg of nicotyrine, 0.5 μg of anabasine

and 1.0 μg of nornicotine give well visible red spots with it. The sensibility can be increased by spraying [8] with 2N H_2SO_4 to such a degree that the 0.1 μg quantity of all the four alkaloids becomes visible (on a dark-brown basis the colour of the nicotyrine and nicotine is carrot-red and anabasine and nornicotine purple-red).

Table 1

The slightest quantities of tobacco alkaloids shown by various detecting reagents, and colour and R_f values of the spots respectively

Alkaloids	Dragendorff reag.		Aniline + BrCN		Benzidine + BrCN		Rf × 100*
	Quantity μg	Spot colour	Quantity μg	Spot colour	Quantity μg	Spot colour	
Nornicotine	1.0	red	0.5	ochre	0.5	purple	18
Anabasine	0.5	red	0.25	pink	0.5	carrot	27
Nicotine	0.1	red	0.1	citrine	0.1	orange	59
Nicotyrine	0.25	red	0.1	orange	0.1	cherry	80

* The given Rf values are the average values of 14—18 chromatograms.

According to KUHN [4] the specific isatin reaction was used for the identification of nornicotine. In this way 0.1 μg of nornicotine gives a well visible blue spot. Proportional with the increase of the quantity of nornicotine the extent of the spot and the depth of its blue colour become more intense. This mode of identification of nornicotine in thin-layer chromatography has not been published before as far as we are acquainted with the literature. Here is its brief description. 0.1 g isatin (Leigh, p.a.) is dissolved in 20 ml glacial acetic acid and filled up with toluene to 100 ml. It is advisable to prepare the reagent before use, although it can be stored in a refrigerator at 4° C for some days. The dry chromatogram is sprayed smoothly with the reagent, then it is put in a drying oven of 100°C for 5—10 minutes. The intense blue colour is sensitive to natural light and fades in some days.

Rf values can not be reproduced in every case, they largely depend on the thickness of the adsorbent layer, the saturation of the tank, the running distance and the used quantity of samples, as well as on the condition whether the alkaloids were applied in a mixture or separately (Fig. 2).

After drying, the thickness of the layers used was about 0.25 mm, the running distance usually 15 cm. Rf values were counted from the spots of 5.0 and 10.0 μg quantities. For a sure identification it is useful to apply on every plate a known concentration of authentic alkaloid solution.

After the methodical pre-experiments the qualitative distribution of alkaloids were determined from the dry leaf samples of various tobacco hybrids. The thin-layer chromatogram of the extracts from seven tobacco samples

can be seen in Fig. 3. A mixture of authentic alkaloid samples was applied on the fourth place of the chromatogram (10 μg of each): a — nornicotine, b — anabasine, c — nicotine, d — nicotyrine. The extracts on place 3, 5, 6 and 7 were freed from chlorophyll, the rest was not. It can be seen well on the chromatogram that for better separation of alkaloids it is useful to remove the chlorophyll from the extract. The extracts of nornicotine-type tobacco hybrids were

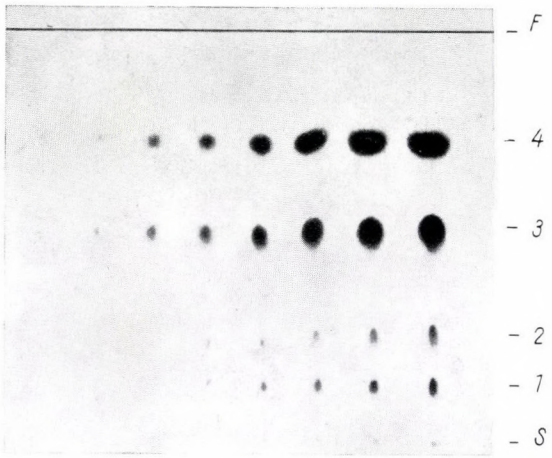

Fig. 1. The thin-layer chromatogram of nornicotine (1), anabasine (2), nicotine (3) and nicotyrine (4). (The applied quantities are given in the text.) Solvent system: chloroform — ethanol = 90 : 10. The time of development 30 minutes. Spot detection with 1% benzidine in ethanol spray and BrCN vapour. S — start, F — solvent front

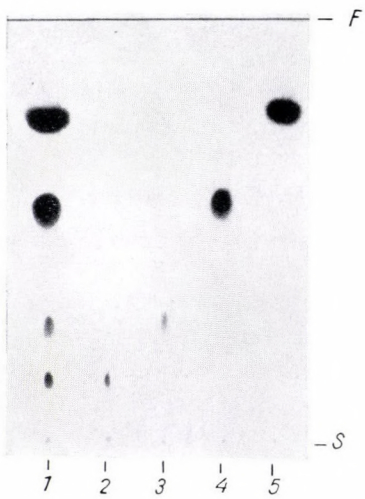

Fig. 2. The thin-layer chromatogram of tobacco alkaloids. 1 — nornicotine, anabasine, nicotine and nicotyrine mixture (10.0 μg of each); 2 — nornicotine (5 μg); 3 — anabasine (5 μg); 4 — nicotine (5 μg); 5 — nicotyrine (5 μg). All other conditions are the same as in Fig. 1

applied on the first three places on the chromatogram and those of nicotine-type in the 5th—8th places on it. The tobacco sample on the first place does not contain nicotine at all, besides a great deal of nornicotine. That could be decidedly established by treatment of the chromatogram with benzidine and BrCN then by UV-light test.

Summarizing we can state that thin-layer chromatography can be effectively applied for the separation and identification of tobacco alkaloids and for a rapid establishment of their qualitative distribution. The quantity of alkaloids in tobacco samples may be approximately estimated as well

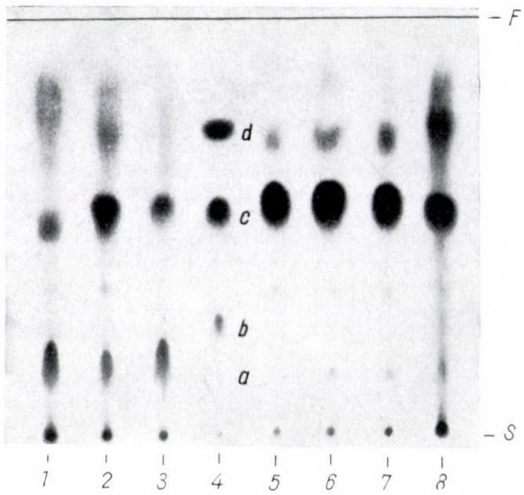

Fig. 3. Explanation in the text

by authentic alkaloids of known concentration which are run in identical conditions i.e. on the same plate. The well-known advantages of thin-layer chromatography are multiplied by the fact that at the separation of tobacco alkaloids the error-possibility coming from volatility owing to the short developing time is reduced to a minimum in relation to paper chromatography.

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UNTERSUCHUNGEN ÜBER DIE ALKALOIDE VERSCHIEDENER NICOTIANA ARTEN UND HYBRIDEN

I. Trennung und Identifizierung von Tabak-Alkaloiden mit Hilfe der Dünnschichtchromatographie

Während unserer Untersuchungen über die Vererbung der Tabak-Alkaloiden wurde bei der schnellen qualitativen und semiquantitativen Bestimmung des Alkaloidgehaltes der verschiedenen Tabak-Arten und Hybriden die Methode der Dünnschichtchromatographie benutzt. Die Dünnschicht wurde nach der üblichen Weise aus Kieselgel G (Merck) mit einer KOH Lösung von 0,5 N hergestellt und 30 Minuten bei 110° C aktiviert. Für die Bestimmung der Empfindlichkeit der Methode und der verschiedenen Entwickler, wurde zuerst aus Reinstoffen von vier Alkaloiden (Nicotin, Nornicotin, Anabasin und Nicotyrin) hergestellte Verdünnungsreihen (0,1—10,0 μg) chromatographiert. Die beste Trennung gab ein Gemisch von Chloroform-Ethanol (90 : 10) als Fließmittel. Von den angewandten Entwicklern bewährte sich am besten das Aufsprühen mit einer Lösung von 2% Anilin in Alkohol, gefolgt von einer Behandlung mit BrCN Dampf. Es wurde festgestellt, daß die Flecken der einzelnen Alkaloide an den mit verschiedenen Reagenten entwickelten Chromatogrammen in UV-Licht charakteristische Fluoreszenz zeigen, wodurch das Identifizieren der in den verschiedenen Tabakmustern vorhandenen Alkaloide stark erleichtert wird. Bei den unter identischen Bedingungen (auf denselben Chromatogramm) gelaufenen Mustern wurden mit Hilfe von Reinstoffen mit bekannter Konzentration auch annähernde quantitative Analysen durchgeführt.

ИЗУЧЕНИЕ АЛКАЛОИДОВ РАЗЛИЧНЫХ ВИДОВ И ГИБРИДОВ ТАБАКА

I. Разделение и идентификация алкалоидов табака при помощи тонкослойной хроматографии

В процессе исследований по генетике табака и наследованию алкалоидов, для быстрого качественного и полуколичественного определения алкалоидов различных видов и гибридов табака, применяли метод тонкослойной хроматографии. Адсорбентный слой изготовляли обычным путем из силикагеля (Мерк) на 0,5 н. растворе КОН, а затем хроматограммы активировали в течение 30 минут при 110°. С целью установления чувствительности метода и различных проявителей сначала хроматографировали смесь четырех стандартных препаратов алкалоидов табака (никотин, норникотин, анабазин, никотирин) различной концентрации (0,1—10,0 μг). Самое лучшее разделение получали в случае применения растворителя хлороформ-этанол в соотношении 90:10 по объему. Из применявшихся проявителей самые лучшие результаты наблюдались при опрыскиванию хроматограмм 2%-ным спиртовым раствором анилина и последующей их обработкой парами BrCN. Установили, что на хроматограммах, проявленных различными проявителями, пятна отдельных алкалоидов в УФ-свете обнаруживают характерную флюоресценцию, что в значительной степени облегчает идентификацию встречающихся в табаке алкалоидов. При помощи стандартных препаратов алкалоидов известной концентрации, хроматографированных в аналогичных условиях, т. е. на той-же хроматограмме, можно провести также и приближенную количественную оценку.

Olga Fejér-Kossey, Budapest II., Herman O. út 15, Hungary

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G. FEKETE

WALDVEGETATION IM GÖDÖLLŐER HÜGELLAND

*

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Verlag der Ungarischen Akademie der Wissenschaften

BUDAPEST

Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója — Műszaki szerkesztő: Farkas Sándor

A kézirat nyomdába érkezett: 1964. IX. 9. — Terjedelem: 12 (A/5) ív, 74 ábra

64.59462, Akadémiai Nyomda, Budapest, V., Gerlóczy u. 2. — Felelős vezető: Bernát György

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Acta Biol. Hung., **15** (3), 257—264 (1965)

EXPERIMENTAL INVESTIGATION OF THE POLLEN OF OLD TREES

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(Received October 11, 1963)

Synopsis

Our comparative experiments concerning the biology of pollens, from the standpoint of the age of the individuals were intended to prove that Phanerophytae of indeterminate growth, similarly to animal species, undergo a definite reproductional involution. In our investigations, in the first place, we endeavoured to find an answer to the question in what degree reproductional involution becomes apparent in the case of old Phanerophytae. Concluding on the degree of involution our aim was physiological investigation of the pollens of individuals of different ages. From quantitative and qualitative data we could conclude on the degree of reproductional involution. In our pollen-biological experiments two viewpoints were taken into consideration: (1) definition of the tube germinating of the pollens originating from young and old trees, their change with age, and (2) intensity of germination of the pollens originating from individuals of various ages and their relationship to the individual's age.

Introduction

Investigations performed on reproduction of living beings of indeterminate growth, their generative organs, respectively, in recent times seem to prove that species of telometric growth undergo a definite reproductional involution in the course of their lives, similarly to that of other living beings. The majority of investigations concerning this question has been performed on animals [3, 4, 6, and others] while data relating to botanical subjects only sporadically occur [13].

In our previous investigations we established that in the case of Phanerophytae the pollens originating from young and old individuals are not physiologically equivalent, i.e. reproductional involution occurs also in old Phanerophytae. In our previous investigations we could draw no conclusions concerning the *degree* of reproductional involution, so we continued these investigations with a greater number of individuals — during another year and on basis of data of years of different ecological conditions we concluded on the degree of involution.

Material and method

Our investigations were performed in the years 1962 and 1963 on the white willow (*Salix alba* L. var. *sericea* GAUD.) originating from the Baja inundation area, 110—120 years old (2 trees), 80—100 y. o. (2 trees), 70—80 y. o. (4 trees), as well as 30 y. o. (2 trees), 20 y. o. (4 trees), and 15 y. o. (2 trees). We established the age of the trees by drilling — by counting

the annual rings — that is, on basis of diameter and circumference according to estimation. Characteristics of the investigation demand that the collected material should originate from biotopes of equivalent ecological conditions; so the experimental material used came from the population of *Salix alba* L. var. *sericea* GAUD. from an area of $^1/_2$ sq. km.

In both years collecting took place at the beginning of blooming (Apr. 20.). In 1962 we performed preliminary investigations on the pollen of two young and two old trees; in 1963 we repeated the investigations on the pollens of eight young and eight old trees, supplementing the morphological data.

While collecting in 1963 we also took the position of the catkins into consideration, therefore we took ten samples off each tree at the following levels: 2—3 m (3 samples) 3—5 m (3 samples), 5—7 m (2 samples), 7—10 m (2 samples). For one sample usually gathered the pollen of 7—10 catkins. The samples were taken from crowns of trees by setting the compass to identical points (south-west). For taking the samples we used test tubes of 1 × 10 cm which we stopped up with cottonwool after having taken the sample and which we placed into a $CaCl_2$ exsiccator on the spot. The exsiccators were stored in a refrigerator at +1 C°.

Germination was performed on special glass plates in hanging drops, in some cases, however, we applied Daniel's method and stained the tubes with haematoxylin and carmin. Germination — after previous experiments and taking the conditions of temperature and sugar concentration into consideration — similarly to the preliminary experiments was chosen so that beside an abundant number of germination the difference between the young and old age groups also appears in a characteristic and definite form. For this reason and for balancing the meteorological deviation of the two years in which investigations took place, we modified the temperature used during germination to 23 C°. Germination, similarly to the experiments in the previous year, was performed in a 10 per cent saccharose solution.

The number of pollens placed in the hanging drop were also taken into consideration, because this — according to literature as well as our own experience — can influence the intensity of tube growth, i. e. the germination of tube growth decisively. Most favourable results were obtained in 0.01 ml. by 800—1,000 pollens.

Germination was investigated for 1.5 hours after collecting and repeated after four days. The average of the results obtained was taken. In evaluating germination and intensity we took the average of ten samples originating from each individual (eight old and eight young trees) from the above mentioned crown levels. We performed parallel germination in each case on young and old trees at the same time. Intensity of germination was registered in the 1, 2, 4, 5.5, 20 and 28th hour. From each sample the tube germination of the maximally germinating pollens was evaluated, so each value is the average tube length of 500 pollens. Tube germination ability was obtained from data of the first hour.

For the intensity of tube germination we elaborated 48,000 pollens, for establishing germinating ability 10,868. For measuring the length of the tube and for determinating germination we used a microscope of type Zeiss Nf.

Results

In our previous investigations [2] we established that a significant difference is found between the tube *germinating ability* of the pollen of young and old samples of *Salix alba* L. var. *sericea* GAUD.

In our investigations performed this year we also found that these differences remained valid. The results — concerning both years of investigations — were summarized in Table 1 and Fig. 1, respectively.

Comparing results of the two years of investigation on basis of the table it was ascertained that germination of the old tree's pollens in the year 1962 yielded 31 per cent, while in 1963 it receeded to 16.26 per cent. Measure of the decrease — expressed percentually — amounts to 52.4 per cent. A similar relapse could be observed in the case of pollens of young individuals too; here the value of germination decreased from 76.6 per cent of the previous year to 42.47 per cent, this decrease amounts to 55.6 per cent.

No conclusions can be drawn as to the relationship between the individual's age and germination of the pollen because of the fluctuating absolute value of the year in question, wherein the meteorological conditions of the respective year are reflected in the first place. Therefore, to establish the percentual ratio of the *sterile pollens* in the old samples of the *Salix alba* L. var. *sericea* GAUD. we evaluated our results relatively, so that the difference existing between the germination of the young and old age groups, and the fact of sterility occurring in old age, respectively, can be displayed significantly.

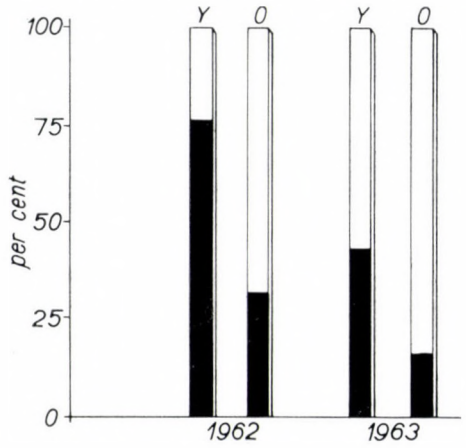

Fig. 1. Absolute values of the germination per cent of young and old age groups during the two years of investigation (Y — young, O — old)

On this basis germination of the old age group yielded 40.6 per cent in 1962 and 38.2 in 1963. Accordingly the relative percentual differences — between the young and old age groups — were 59.4 and 61.8 per cent. The relative percentual differences, i.e. values of the pollen germination of old age groups, showed an insignificant deviation during the two test years, *only* 2.4 *per cent.*

Table 1

Per cent of pollen germination of old and young age groups during the years of investigation of 1962 and 1963

Year of investigation	Age group	Total number of pollen investigated	From the germinated pollen		Per cent of tube germinated pollens
			not germinating tubes	germinating tubes	
1962	Young	1660	1273	387	76.6
	Old	1736	538	1198	31.0
1963	Young	5842	2606	3236	42.4
	Old	5026	820	4206	16.2

The values of germination in the pollens of old individuals and their annual changes are presented in Fig. 2.

Our further investigations — similarly to those of the previous year — were extended to the *intensity of the tube's growth*, as well as its changes during germination. Furthermore we investigated the time deviation of the tube length growth between the pollens of different age groups, that is, the measure of *latent time*.

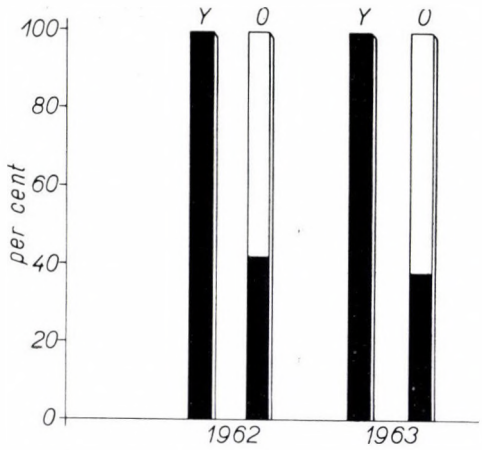

Fig. 2. Relative value of the germination per cent of old age groups during the two years of investigation. (Y — young, O — old)

Examining the intensity of germination of the age groups, we pointed out the results obtained in the previous year, according to which there is a definite connection existing between age of the individual and intensity of germination of its pollens; similarly to germination of pollens of different age groups.

Our results are presented in Table 2 from which it can be established that, similarly to the tube germination of the pollens, intensity of tube germination of pollens of both age groups showed a definite *relapse* in the present year (Table 2).

While in consequence of favourable ecological conditions of the year 1962, the rate of the tube growth of the pollens displayed a high value already in the first hour (young age groups 124 μ, old age groups 68.5 μ), the rate of growth in the present year only reached similar values in the 4—5th hour. However, in consequence of the tubes' growth slowing down, the *difference* of intensity appearing in tube's length growth of pollens of both age groups was even more striking (Fig. 3). In this year's investigations the tubes terminated their growth in the 28th hour in consequence of the high degree of decrease of intensity in the tube's growth, whereas in the previous year this occurred already in the tenth hour.

Similarly to the previous investigations of 1962, this year a temporal deviation can also be observed in the intensity of the tube's growth of the pollens of young and old age groups — as represented in the Table. In this year the length of the *latent time* became even greater, since in consequence of the slowing down of the tube's growth it *lengthened* significantly. As a result of this, the difference arising between the rates of the pollen's tube growth of

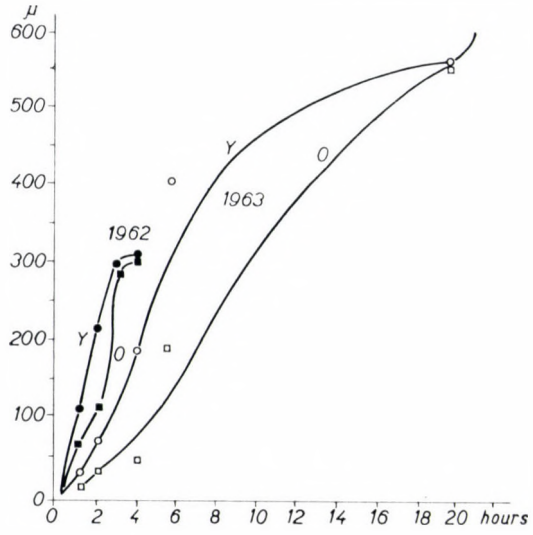

Fig. 3. Intensity of the tube growth of pollens belonging to the young and old age group, during the two years of investigation. (Y — young, O — old)

both age groups appears to an ever higher degree. It is characteristic of the degree of difference, that while the rate of the tube length growth of pollens of old and young age groups in the previous year balanced already in the third hour, this year the difference was compensated only in the 20th hour.

Table 2

Intensity of pollen germination of old and young age groups during the years of investigation of 1962 and 1963

Year	Age group	Registrations in hours						
		1	2	3	4	5.5	20	28
		Average length of tubes in μ						
1962	Young	124.0	234.6	280.7	292.0	—	—	—
	Old	68.5	130.4	282.1	317.1	—	—	—
1963	Young	33.2	67.9	—	185.4	412.4	577.2	1150.0
	Old	11.7	29.8	—	40.9	192.1	576.4	1142.0

Subsequently we performed morphological investigations on the pollens of young and old individuals. No significant morphological deviations could be observed on the pollens of young and old trees. Dimension of the pollen is 15 × 20 μ. At the termination of growth the length of the tube yielded a value of 1150 μ an average by old as well as young age groups. In these cases a small per cent of longer tubes occurred too their length approaching 2,200 μ. At termination of growth a knob-like widening appeared on the end of the tube which reached a dimension of 15 × 15 μ. In the lengthened section generative cell nuclei could be well observed. The vegetative cell nucleus of the tube stained intensively during growth with carmin, while subsequently at the widening of the tube it gradually disappeared. We also observed the cellulose degeneration of the growing tubes' protoplasm which is described by STRASBURGER, OLIVER, BENSON and others [9]. The degenerational phases are repeated regularly in the tubes of the pollens of the young as well as old trees. On the tubes which have terminated their growth, generally 4—5 thickened sections can be noticed (degenerated phases), length of the tubes being 15—18 per cent, 49—54 per cent, 65—70 per cent and 95 per cent.

Discussion

Concluding on the degree of reproductional involution, we took germinating ability of the pollens as a measure of sterility. In the course of the previous investigations [2] the question arose whether or not difference in germination could be ascribed to the various ecological conditions — in the first place to meteorological factors. It is well known that germinating ability of the pollen-grains as well as intensity of tube germination, and their absolute value respectively change annually [14] and that the reason for this can be sought for in the above-mentioned factors.

The fact that the absolute values of our investigations performed in the years 1962 and 1963 — comparing the equivalent age groups of the two years — show essential deviations, can be ascribed to the afore said. The explanation for this is that, as we know from literature [12 and others], differentiation of the flower bud takes place in summer time (from June to September) and so the organs in embryonal condition (blossoms) are considerably exposed to ecological factors (frost damage, etc.). On this basis it seems to be proved that the very cold winter of 1963 — contrary to the milder one of 1962 — significantly modified the absolute results. We previously expounded the degree of difference in detail. We also described the relative germination values of the old age groups during the two years and established that the difference appearing — during the two years of investigation — decreased to a minimum of 2.4 per cent. Therefore it can be taken for certain that in the case of *Salix alba* L. var. *sericea* GAUD. germination of the pollens decreases significantly with

age, i.e. in line with this, the number of sterile pollens increases and ecologicai effects prevail only in the absolute values of germination.

To prove our results concerning the connection between age and germination, we must refer to the work of SANDSTEN [13] in which similar results are reported on germination of pollens of young and old apple-trees.

Referring to the mechanism of germination, onset of tube growth and substances activating intensity of tube growth, data of literature substantially differ, although numerous works deal with the qualitative and quantitative analysis of these substances [1, 7, 8, 10, 14 and others].

It should be mentioned that overall, detailed investigations concerning the individual's age and germination mechanism of the pollens are missing. Our own investigations draw attention to the fact that in germination mechanism the pollens originating from individuals of various ages undergo fundamental changes with age.

It is still too early to give explanations of our results and neither can we make assumptions because as we have already mentioned, data taking the age of the individual into account are not found in literature. In the first place it becomes necessary to perform qualitative and quantitative investigations of the proper substances — enzymes, vitamins, etc. — of pollens; as well as analysis of the stigma-secretion substance which, as is well known, greatly influences onset and intensity of the tube growth [11 and others]. Our work intending to solve these problems is in progress.

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EXPERIMENTELLE UNTERSUCHUNG DES POLLENS ALTER BÄUME

Vergleichende Untersuchungen über das Lebensalter zeigten auf dem Gebiet der Pollenphysiologie, daß indeterminiert wachsende Holzgewächse ebenso eine Fortpflanzungsinvolution durchmachen wie die Tierarten. Als Grundlage der Arbeiten diente die physiologische Untersuchung des Pollens verschieden alter Individuen. Aus den qualitativen und quantitativen Befunden wurde auf das Maß der Fortpflanzungsinvolution geschlossen. Bei den Pollenphysiologischen Untersuchungen beachteten wir: (1) Die Fähigkeit zum Auswachsen des Pollens von verschieden alten Bäumen, bzw. die Änderung in der Fähigkeit des »Keimens« bei verschieden alten Individuen. (2) Die Intensität des Auswachsens von Pollen verschieden alter Individuen, bzw. die Änderung der Intensität mit dem zunehmenden Lebensalter.

ЭКСПЕРИМЕНТАЛЬНОЕ ИЗУЧЕНИЕ ПЫЛЬЦЫ СТАРЫХ ДЕРЕВЬЕВ

В результате физиологических исследований пыльцы, произведенных с учетом возраста особи, авторы получили доказательства, что древесные растения неопределенного роста — подобно животным видам — также обладают определенной инволюцией размножения. Исследования проводились в первую очередь для выяснения того, в какой именно степени выражена инволюция размножения у старых древесных растений. Выводы о степени инволюции сделаны на основании качественных и количественных данных, полученных в процессе физиологического исследования пыльцы особей различного возраста. При физиологических исследованиях пыльцы обращали внимание на следующее: (1) Установление способности прорастания пыльцевых трубок старых и молодых деревьев, и изменение этой способности в зависимости от возраста; (2) Интенсивность прорастания трубок пыльцы особей различного возраста, и связь интенсивности прорастания с возрастом.

Miklós Burg, Budapest IX., Üllői út 93, Hungary.

Acta Biol. Hung., **15** (3), 265–269 (1965)

QUANTITATIVE STUDIES OF THE NUCLEOPROTEIN CONTENT OF ERYTHROCYTES FROM HEALTHY AND VIRUS-INFECTED CHICKEN

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(Received October 13, 1963)

Synopsis

The authors have studied by cytophotometry the differences in DNA and protein content in the peripheral erythrocytes from normal hens, chicken embryos and from chickens infected with avian erythroblastosis virus. There were no quantitative differences in DNA content but as to protein content it was found that the nuclei of the erythrocytes from the virus-infected animals contained much more protein than the nuclei of the erythrocytes from normal or embryonic chickens.

Introduction

In various cases of myeloid and lymphoid leucaemia, leucosis, the quantities of deoxyribonucleic acid (DNA) and of the basic protein (histon) contained in the nuclei of the pathological cellular elements have been analyzed by numerous authors [4, 10]. In avian erythroblastosis virus-infected young erythrocytes in different stages of development are demonstrable both in the bone marrow and in the peripheral blood, beside the other haematological changes [5, 6]. The submicroscopic structure of the virus-infected cells has also been described [2].

The question to be elucidated is: does the virus-infection produce any quantitative change in the DNA and protein contents of the nuclei of erythrocytes?

Material and method

Blood smears of virus-infected, about 4-month old Leghorn chickens, normal adult (2,500 g) Leghorn hens and 12-day old Leghorn chicken embryos were examined. To facilitate comparison with the cytophotometric measurements, smears were prepared also of the liver of healthy hens.

The smears were dried at room temperature and stored at $+4$ C° until use. Fixation in 10 per cent neutral formol lasted 10 minutes, and was followed by washing with tap water for 20 minutes. The smears were stained with May-Grünwald-Giemsa's stain. Some smears were tested by the Feulgen reaction (duration of hydrolysis: 2, 4, 6, 8, 12, 14, 18 and 22 minutes) and by the ninhydrin-Schiff reaction.

The measurements were made with a cytophotometer prepared by us (RAPPAY, unpublished) at 560 mμ, measuring 25 nuclei in every case.

Results

May-Grünwald-Giemsa staining

The hen and chicken embryo blood smears showed normal cellular pattern. The nuclei of the hen erythrocytes appeared pyknotic. In the blood smears of the virus-infected chicken a great number of erythroblasts were visible, including many dividing ones (Fig. 1).

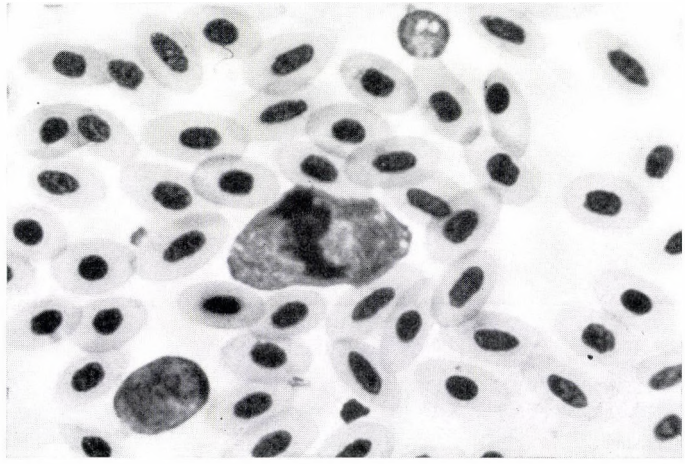

Fig. 1. Smear from the peripheral blood of virus-infected chicken. May-Grünwald-Giemsa staining, × 400

Feulgen's reaction

After 12 minutes of hydrolysis we measured diploid DNA content in the nuclei of liver cells, embryonic and virus-infected erythrocytes; in the nuclei of the old hen's erythrocytes, on the contrary, we found hypodiploidism (Fig. 2). After 12-minute hydrolysis were found less, after 4-minutes of hydrolysis there were more tetraploid nuclei in the smears from the virus-infected animals (Fig. 3). At the same time, the amount of the bound stain was hardly less in the diploid nuclei than in the nuclei of the smears hydrolyzed for 12 minutes.

Ninhydrin-Schiff reaction

The nuclei of the hen-erythrocytes are pale, and therefore they absorb little light. The most intense reaction was shown by the nuclei of the virus-infected animals. The values of the chicken embryo's erythrocytes nuclei are between these two extremes (Table 1).

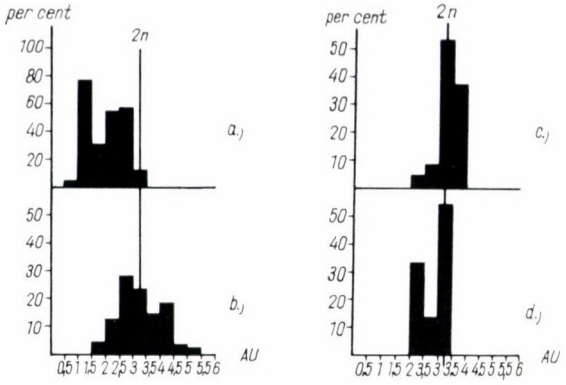

Fig. 2. Distribution curves of Feulgen measurements

a — Erythrocytes of old hen
b — Erythrocytes of erythroblastosis virus-infected chicken
c — Erythrocytes of chicken embryo
d — Liver cells of adult ben

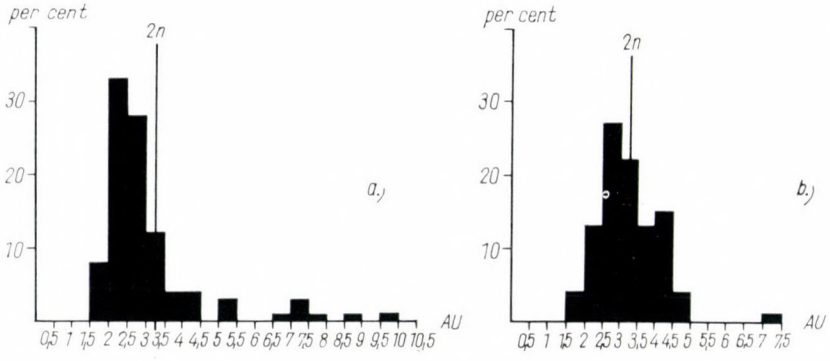

Fig. 3. Feulgen measurements on erythrocytes of erythroblastosis virus-infected chicken after HCl-hydrolysis for 4 and 12 minutes respectively

Table 1

Cytophotometric measurements of ninhydrin-Schiff reaction

Object	AU
1. Old hen	0.437±0.013
2. 12-day old chicken embryo	0.607±0.051
3. Erythroblastosis	0.775±0.070

Discussion

The results of these experiments may be discussed from two points of view. One of them is the quantitative and qualitative behaviour of the DNA in the erythroblasts of the virus-infected animals, compared with the normal liver cells and erythrocytes nuclei of different ages. It can be stated that the virus infection produced no measurable change in the DNA content of the erythroblasts. This statement is in agreement with the results of the authors mentioned in the introduction [4, 10], who found the same data on different objects. According to this, the DNA content is always normal in the pathological cellular elements of the patients suffering from myeloid and lymphoid leucaemia.

Though the quantitative change of the DNA was not observed in our case, the total deoxyribonucleoprotein complex might be altered by a virus infection. This is indicated on the one hand by the behaviour of the hydrolysis curves of the erythrocytes examined by us, on the other hand by the results of the ninhydrin-Schiff test. As far as the former is concerned, the intensity of colour of the virus-infected cell nuclei was almost the same after a short (4 minutes) Feulgen hydrolysis, as after 12 minutes hydrolysis. The shape of the hydrolysis curves, especially of the ascending part, is quite different from that of the other nuclei, although the maximum time required for attaining the optimal colour intensity is about 12 minutes. Such differences in the hydrolysis curves have been found in other cases, too [8].

When evaluating the results obtained by the ninhydrin-Schiff tests, the first thing to clarify is whether this method, serving to demonstrate the free alpha amino groups of the amino acids built in the protein chain, is suitable or not for the quantification of the nucleoproteins. The specificity of the method is unquestionable [1, 3], and under rigidly controlled conditions it can be used for quantitative purposes, too [7, 9]. We must consider the reaction for a method apt to a general demonstration of the proteins.

As to the results of measurements now obtained, it is to be pointed out that the nuclei of the erythrocytes of the animals infected with the erythroblastosis virus contain much more protein demonstrated by the ninhydrin-Schiff reaction than those of the normal adult or normal embryonic chickens. This once again harmonizes with the results of ZAMPORI, PERUGINI and coworkers, quoted earlier and informing us about the increase of the quantitiy of basic proteins in their objects.

On the basis of the experimental results obtained it seems interesting to clarify whether the virus infection would cause other changes demonstrable by quantitative cytochemical methods, in the cellular elements in question.

Acknowledgements

The authors are pleased to be able to express their thanks also this way to F. Csemniczky for his valuable technical assistance and to Dr. P. Kertai for his courtesy in placing at our disposal the blood of virus-infected animals.

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QUANTITATIVE UNTERSUCHUNGEN ÜBER DEN NUCLEOPROTEID-GEHALT DER ERYTHROZYTEN VON GESUNDEN UND MIT VIREN INFIZIERTEN HÜHNERN

Verfaßer untersuchten mit der cytophotometrischen Methode den DNS- und Eiweißgehalt der peripheren roten Blutzellen von normalen Hühnern, Hühner-Embryonen und von, mit avian erythroblastosis Viren infizierten Hühnern. Im DNS-Gehalt konnte kein Unterschied festgestellt werden. Ein Unterschied zeigte sich im Eiweißgehalt; die Kerne der roten Blutzellen von infizierten Tieren enthielten bedeutend mehr Eiweiß als die der normalen und embryonalen Hühner.

КОЛИЧЕСТВЕННОЕ ИЗУЧЕНИЕ СОДЕРЖАНИЯ НУКЛЕОПРОТЕИНОВ В ЭРИТРОЦИТАХ ЗДОРОВЫХ И ЗАРАЖЕННЫХ ВИРУСАМИ ЖИВОТНЫХ

Авторы методом цитофотометрии исследовали разницу в содержании белка и ДНК в периферических красных кровяных клетках здоровых кур и куриных эмбрионов, а также цыплят зараженных вирусом авиан-эритробластоза. Разницы в содержании ДНК не наблюдалось, зато в содержании белка имеется количественное различие, а именно: в ядрах кровяных клеток животных, зараженных вирусом эритробластоза, имеется значительно больше белка, чем в ядрах кровяных клеток здоровых старых или эмбриональных животных.

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Acta Biol. Hung., **15** (3), 271—284 (1965)

HISTOCHEMICAL AND ELECTRON MICROSCOPICAL STUDY OF THE ADRENAL MEDULLA OF THE GRASS SNAKE (NATRIX NATRIX)

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(Received January 6, 1964)

Synopsis

Concerning the structure of the adrenal medulla of the grass snake no data can be found in the literature of either optical or of electron microscopy. Using histochemical and electron microscopic methods, the authors have demonstrated three cell types in the adrenal medulla of the grass snake: (1) noradrenaline cells with large catecholamine granules, about 2500 Å in diameter, which contain noradrenaline; (2) adrenaline cells characterized by the presence of the so-called "small catecholamine granules", 1250 Å in diameter, which contain adrenaline. Both cell types have a light cytoplasm poor in cell components. (3) The so-called "dark cell type", in which, in addition to both the large and the small types of catecholamine granules the authors also observed precursor dopamine granules 0.5—1.5 μ in size, Golgi apparatus, multivesicular bodies, a few mitochondria, several Palade granules, and vesicular ergastoplasm.

Introduction

Although the results obtained by light and electron microscopical methods are not in keeping with each other or with the histochemical observations, more and more evidence is accumulating in favour of the theory that in the adrenal medulla of the grass snake two cell types exist side by side. Several authors have investigated various experimental animals, which makes the evaluation of their results difficult. Because of their close morphological connection, cortical cells have also been described as medullary cells in many cases. The interesting works of Yates and co-workers [17] represent another step forward. They were the first workers to reconcile the results obtained by different methods, and they described adrenaline and noradrenaline cells showing distinct differences in granulation. On the basis of their phase and electron microscopic findings, they established that the zone close to the cortex mostly contains noradrenaline cells with large granules (2,000 Å), but in the middle of the gland adrenaline-producing cells containing small granules (of 700—1000 Å size) predominate.

These results of Yates were confirmed by the fluorescence microscopical investigations of Eränkö [5], who found that in the gold hamster the noradrenaline cells were concentrated in the peripheral region of the adrenal medulla. The experiments of Hagen and Barnett [9] (fractionation of granules with

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the ultracentrifuge) also corroborate YATES' theory. Those authors found that the noradrenaline content of the medulla was concentrated in the large granules, and the small ones were rich in adrenaline. As there are only very few data available on this problem in the lower vertebrates [3, 4, 14], we have carried out a series of experiments on amphibians, reptiles and birds, in order to obtain some new data on the phylogenetic aspects of the question. The present work deals with (the analysis of) the histochemical and submicroscopic structure of the adrenal medulla of the grass snake *(Natrix natrix)*, with special regard to the identification of the adrenaline- and noradrenaline-producing cells. Our attention was drawn to this problem by WASSERMAN's [14] histochemical study of the adrenal medulla of the snake *(Xenodon Merremii)*, in which he easily distinguished, even anatomically, the groups of adrenaline- and noradrenaline-producing cells. He succeeded in doing the same also by histochemical and biochemical methods.

Material and method

In our experiments we used specimens of the grass snake *(Natrix natrix)* collected in May. The animals were anaesthetized with ether, and their adrenals removed after decapitation. Determination of the adrenaline and noradrenaline contents was performed by the Euler-Hamberg method [8] as modified by us [13].

Light microscopy : For the histochemical demonstration of adrenaline and noradrenaline bhe adrenal glands were fixed according to KLEIN [10] in Ortsche's solution (9 parts potassium 3sichromate in Müller's solution + 1 part 40 per cent formol) for 24 hours, then in Müller's tolution for 48 hours. Following washing, dehydration and embedding in paraffine, sections of about 8—10 μ thickness were made of the glands. Haematoxylin-eosin staining was used for general study.

Electron microscopy : For electron microscopic study the adrenal glands were cut into pieces of about 1 mm in size and prefixed for 48 hours in formol buffered with phosphate to pH 7.8 [2]. This was followed by fixation according to PALADE [12] in OsO_4 for one hour. The material was dehydrated for 5 minutes each in 30, 40 and 50 per cent alcohol; for 10 minutes each in 60, 70, 80 and 90 per cent alcohol and for 30 min. in absolute alcohol. Embedding in Araldite. Polymerization of the material was carried out at 56° C for 48 hours, and cutting with glass knives on an LKB Ultrotome. The sections showed gray interference colours in fluorescent light. They were stained with "lead". An ELMI D_2 (Zeiss-Jena) electron microscope was used.

Results

Since there were no data available on the adrenaline and noradrenaline contents of the grass snake adrenal, first they were determined chemically on 5 tissue samples. It was found that 1 g fresh adrenal tissue contained 650 μg adrenaline and 760 μg noradrenaline, which corresponds to a ratio of 46/54, as compared with the values known for mammals (e.g. for the rat 80/20); this noradrenaline content is relatively high.

Histochemical studies

The first micrograph (Fig. 1) represents the results of the histochemical studies. The cortex *(C)* contains a large number of nuclei and the cytoplasm

of these cells is light because it did not react with potassium bichromate. The chromaffin tissue of the adrenal reacting with the potassium bichromate appears dark. Some of the chromaffin cell groups are scattered in the cortex as dark islets *(AC)*, others lie outside the cortex and form a "ribbon". The latter contain hardly any cortex cells, only light yellow, roughly granulated noradrenaline *(NAc)* cells with nuclei poor in chromatin and dark green, finely granulated adrenaline cells *(Ac)* with nuclei abundant in chromatin.

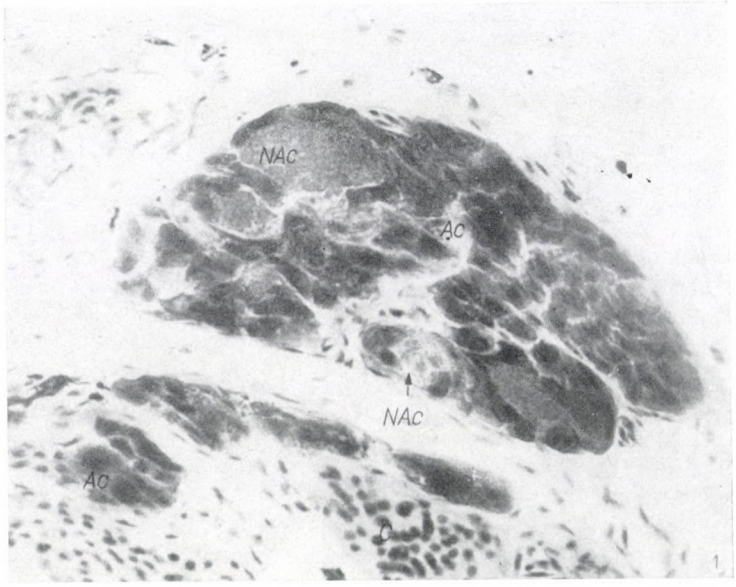

Fig. 1. Photomicrograph of a cross-section of the adrenal of the grass snake. The noradrenaline cells *(NAc)* are light, the adrenaline cells *(Ac)* dark. The noradrenaline cells have roughly granulated cytoplasm, and their nuclei are poor in chromatin. The cytoplasm of the adrenaline cells is finely granulated, and their nuclei rich in chromatin.
Histochemical method of Klein. Haematoxylin-eosin, × 250

Ultrastructure of the noradrenaline cells

The noradrenaline cells may be observed lying side by side (Fig. 2) or beside the adrenaline *(Ac)* or cortex cells and are separated by a wide basal membrane *(bm)* but in some cases by a cell membrane (Fig. 2). Their cytoplasm is of the "light type" and rather poor in cell organelles. The most striking components in the cytoplasm are the catecholamine granules of great density most of which are spheroid or ellipsoid (Figs. 2, 3). The granules are usually surrounded by a membrane although there are some without a membrane. In some cases the membrane surrounding the granule sticks closely to it, in others a light, electron permeable region can be found between the granule

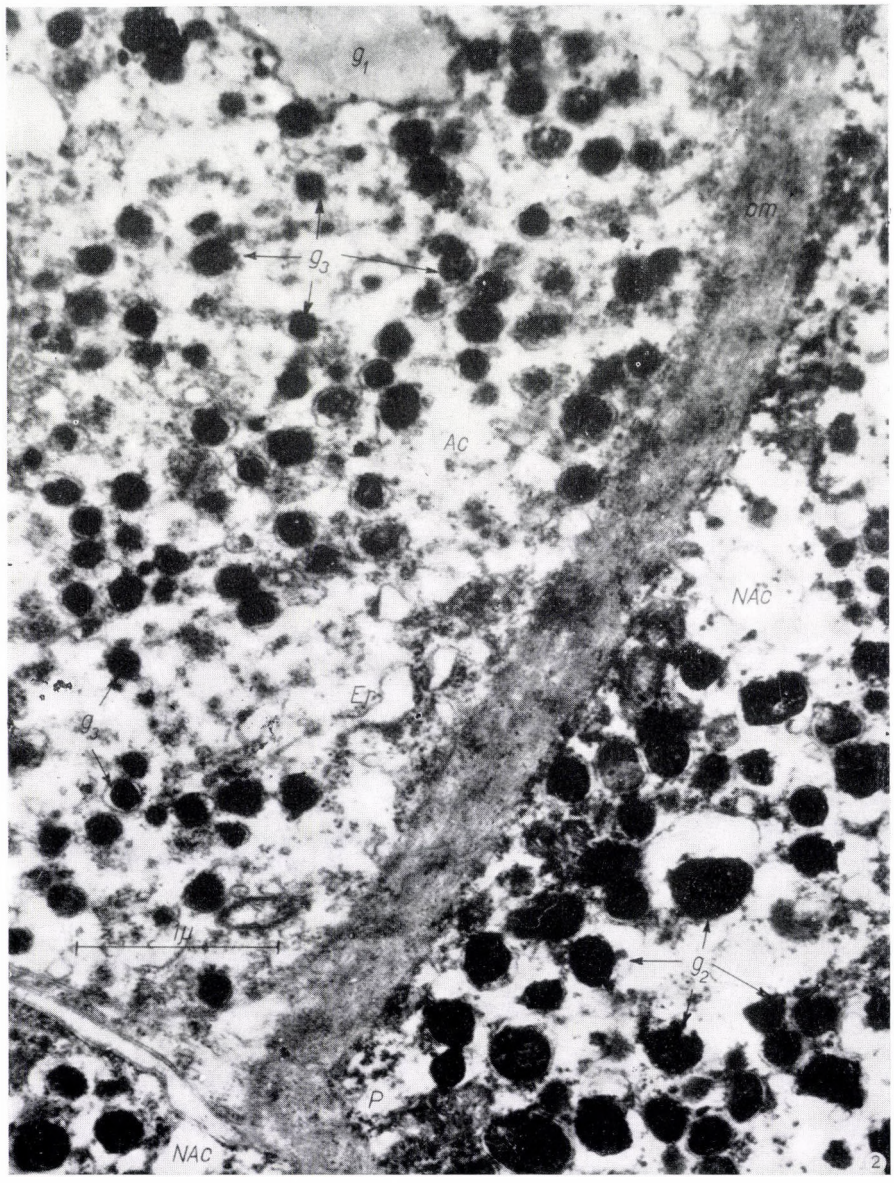

Fig. 2. Electron micrograph of the adrenal medullary cells of the grass snake. At the top on the left there is an adrenaline cell *(Ac)*. In the cytoplasm many small granules (g_3) can be seen. Their diameters are about 1,250 Å. In the cell there are also large precursor granules (g_1) and expanded ergastoplasm *(Er)*. The neighbouring noradrenaline cells *(NAc)* have very light cytoplasm, which is poor in cytoplasmic organoids, but contains many large catecholamine granules (g_2). (Diameter, about 2,500 Å)

and its membrane. The distribution of the granules in the cytoplasm is usually even, however, light areas without granules can sometimes also be seen. The diameter of the catecholamine granules in the noradrenaline cells is about 1500—3500 Å. In the noradrenaline cells we failed to demonstrate mitochondria, Golgi apparatuses and rough surfaced ergastoplasm, however, we succeeded in observing Palade's granules and precursor granules which were 0.5—1.5 μ large in size (Fig. 3) in greater or smaller numbers.

Ultrastructure of the adrenaline cells

It is characteristic of the adrenaline cells (Figs. 2, 3) that the diameter of their catecholamine granules is about 1200 Å, which is about half of the value for the granules of the noradrenaline cells. Beside the granules of average size there are others in the adrenaline cells which vary in size from 500 to 2000 Å. The other submicroscopic characteristics of the adrenaline cells are generally the same as those of the noradrenaline cells, except that in the adrenaline cells the vesicular type of ergastoplasm can often be observed.

Dark cell type

Besides the two cell types mentioned above the adrenal medulla of the grass snake contains also a third type, the submicroscopic properties of which show considerable deviations from the others. Their most striking feature is the dark character of the cytoplasm (Figs. 4, 5, 6) which may be due to (1) the high content of Palade's granules, (2) the large number of catecholamine granules (including precursor granules), (3) to the presence of other cell components (Golgi apparatus, mitochondria, etc.).

The structure of the nucleus is nonhomogeneous. Besides the fine granulated parts of the karyoplasm roughly granulated areas may be observed and others with low electron density (Figs. 4, 5). The submicroscopic properties of the catecholamine granules are the same as those of the cells already described above, however, their diameter varies between 500—3,000 Å.

In addition to the typical catecholamine granules, there are also granules with a diameter of 0.5—1.5 μ, which are typical components of the cells with dark cytoplasm. On the basis of their electron microscopic structure, these granules could not be ranged into a uniform group, therefore the ones with identical properties were classified into one group.

A definite type of these precursor granules is situated in the lumen of the ergastoplasmic cisternae (Fig. 5) and has no membrane of its own. In the lumen of some of the cisternae sometimes several granules may be found. The inner structure of the granules consists of fine, microgranular elements, and contains a strongly electron-scattering substance. In some cases these

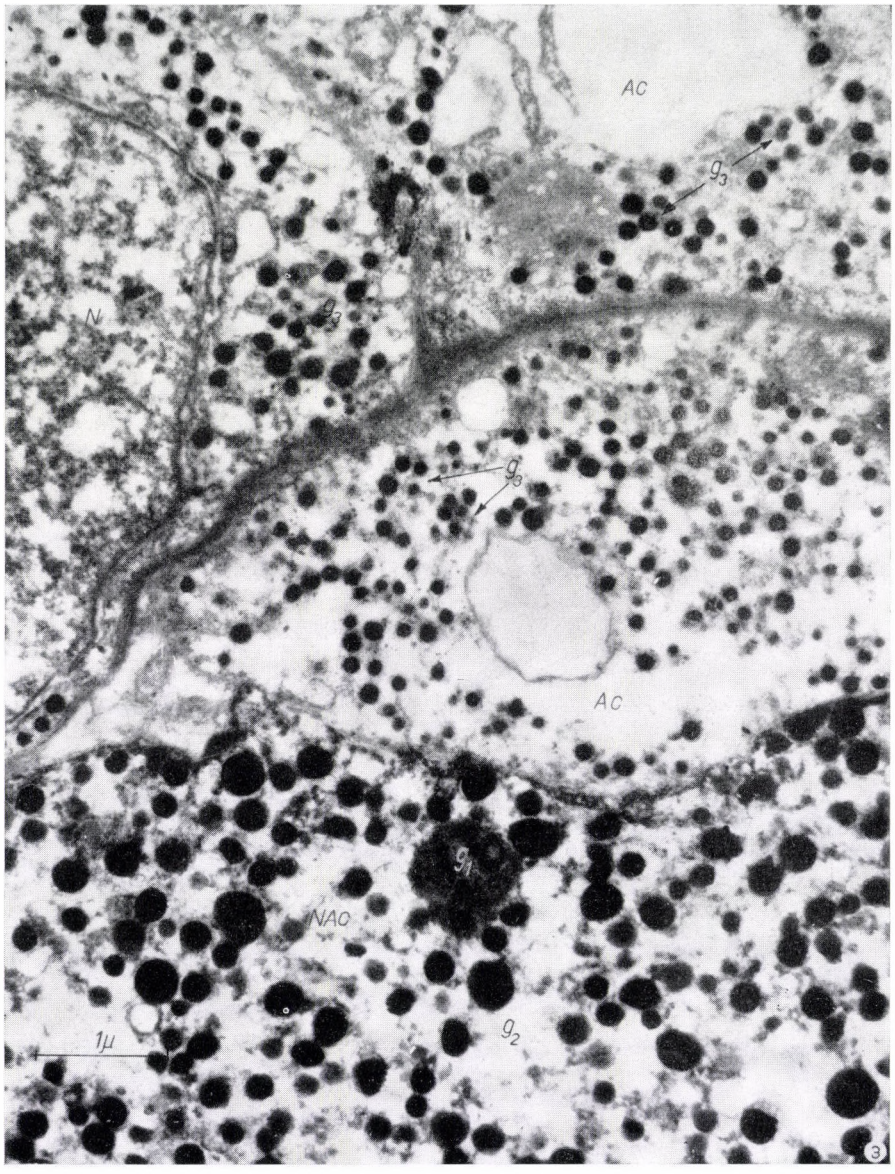

Fig. 3. Electron micrograph of the adrenal medulla of the grass snake. Both the adrenaline *(Ac)* and the noradrenaline cells *(NAc)* have fairly light cytoplasm, and besides the catecholamine granules (g_2, g_3) only a nucleus *(N)*, large precursor granules (g_1) and vesicular ergastoplasm *(Er)* can be seen in the cell. × 18,000

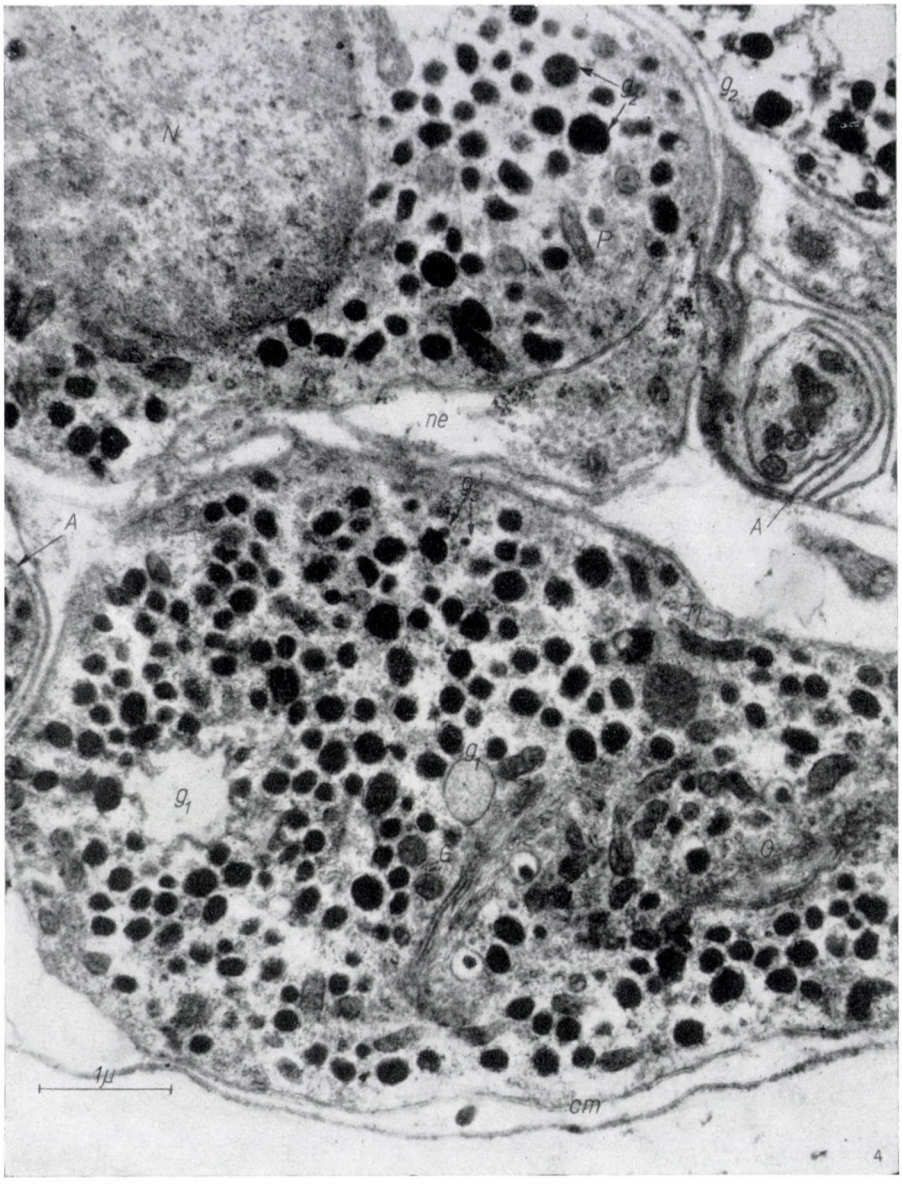

Fig. 4. "Dark" cells from the adrenal medulla of the grass snake. In the cytoplasm many Palade granules *(P)*, catecholamine granules of various sizes *(g_2, g_3)* a large precursor granule *(g_1)*, mitochondria *(M)* and a well-developed Golgi-apparatus *(G)* can be seen. Among the cells there are cross-sections of axons *(A)*. × 18,000

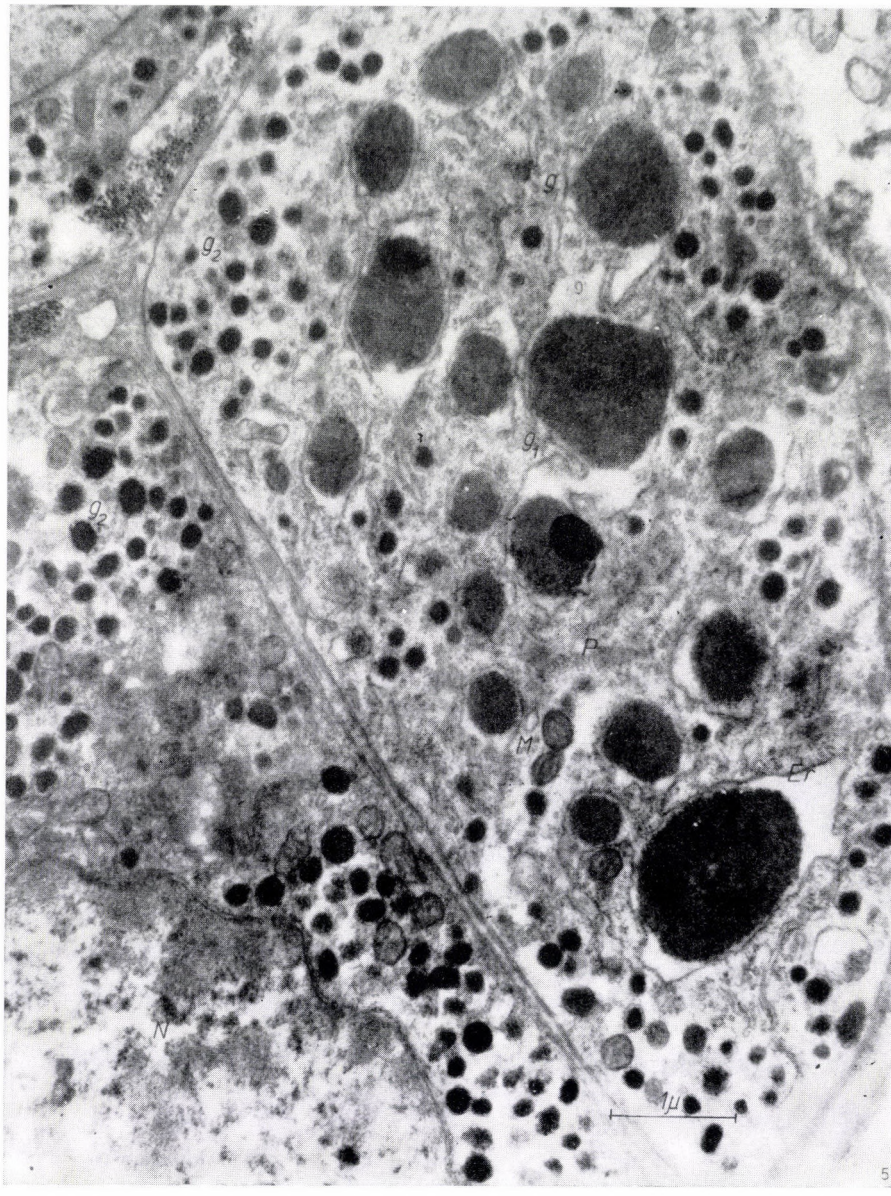

Fig. 5. "Dark" cells with large precursor granules varying in size between 0.5 and 1.5 μ (g_1). They are usually surrounded by the membrane of the vesicular ergastoplasm (*Er*), show varying electron density, and have a finely granulated inner structure. Numerous catecholamine granules (g_2), a few mitochondria (*M*) and many Palade granules (*P*) may be seen in the picture. In the lower part of the picture, on the left, there is part of a nucleus (*N*) and close to it numerous catecholamine granules (g_2) can be observed. ×18,000

granules scatter the electrons completely so that their electron density reminds of that of the catecholamine granules. In a few of the ergastoplasmatic cisternae the precursor and the catecholamine granules occur together (Fig. 5), which is indicative of their close morphogenetic relationship. The precursor granules marked with g_1 (Figs. 2, 4) seemed to be surrounded by membranes of their own. They usually have an irregular shape, contain a substance which scatters (absorbs) electrones poorly and are often in close morphological contact with the catecholamine granules.

The third group of the precursor granules is formed by granules which have regular ellipsoid shape, are bound by a membrane and show varying inner density. They may be observed in isolation near the Golgi apparatus (Fig. 4) and in the cytoplasm (Fig. 6).

The number of mitochondria is comparatively low. Depending on the plane of section, their cross-section is elongated or oval, and they contain few cristae.

The Golgi apparatus may often be seen in this cell type (Figs. 4, 6). Especially noteworthy (Fig. 4) is the mass, consisting of lamellar and vesicular components, in which at either pole there is a small catecholamine granule surrounded by a membrane and a light area.

The ergastoplasm is generally of the vesicular type and is fairly rich in Palade granules (Fig. 6); its quantity varies on a wide range, probably depending on the developmental stage of the cell. For example, in the cells (Fig. 4) the ergastoplasm is practically absent, while in others (Fig. 6), it is well developed.

Discussion

The aim of most of the morphological and histochemical investigations into the secretory activity of the adrenal medulla has been reliable identification of the two cell types which produce adrenaline and noradrenaline, respectively. As early as 1950, and later in 1954 EULER [7] expounded his theory that these two hormones are produced in different conditions, and it could hardly be imagined that both were synthetized in one and the same cell. This theory was experimentally proved first by BÄNDER [1], and by ERÄNKÖ [5]. Working with Altmann's staining method, BÄNDER isolated on the one hand picrinophylic (light), and on the other hand fuchsinophylic (dark) cell types, and claimed that the "P" cells produced adrenaline, and the "F" cells noradrenaline. Fluorescence microscopic investigations by ERÄNKÖ [5] of the adrenal medulla of gold hamster represented a further step toward the identification of these two cell types. Yet, on the basis of his electron microscopic observations on the rat, LEVER [11] only mentions light and dark cell types, and does not indicate them as the respective places of production of adrenaline

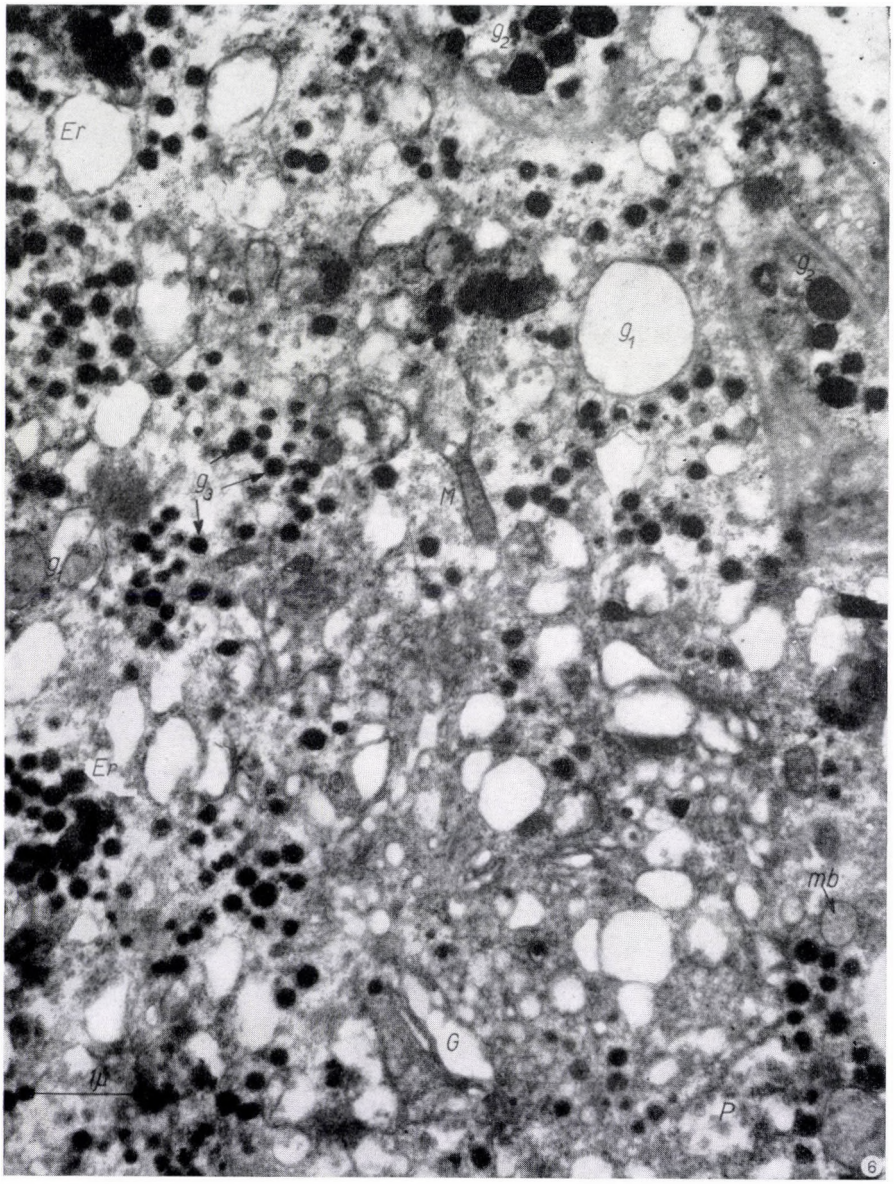

Fig. 6. "Dark" cell, in which a few large precursor granules can be observed, but both small and large catecholamine granules *(g_2 g_3)* occur together. The well-developed ergastoplasm *(Er)* is of the vesicular type. The Palade granules *(P)* in the cytoplasm are widely scattered. Besides the Golgi apparatus *(G)* there are mitochondria *(M)*, and multivesicular bodies *(mb)* in the dark type of cytoplasm. × 18,000

and noradrenaline. When analyzing this question WETZSTEIN [15] puts forward an interesting theory, according to which the "main cells" of the adrenal medulla of the mouse, cat and guinea pig produce adrenaline, however, at the same time he considers the production of noradrenaline by the so-called "Nebenzellen" (accessory cells) disputable. It is surprising that in contrast to his earlier fluorescent microscopical findings ERÄNKÖ's later electron microscopical study of the rat [6] distinguishes not two, but three different cell types; however, he does not indicate the place of production of noradrenaline and adrenaline. Neither does he consider it impossible that this third cell type be of cortical origin. Starting partly from the fluorescence microscopical findings of ERÄNKÖ, and the histochemical data of WOOD [16], YATES and co-workers [17], using the electron microscope, also described two cell types with different granulation in the adrenal medulla of the gold hamster. On the basis of electron microscopical, phasecontrast microscopical and histochemical observations they regarded the cells containing mostly small granules (700—1000 Å) as adrenaline-producing cells and the peripheral cells containing larger granules (1,000—3,500 Å) as noradrenaline-producing ones. This observation is in good agreement with our experimental findings. With the histochemical method of KLEIN [10] we observed solitary medullary cells in the so-called "medullary margin" and among the cortical cells of the adrenal medulla of the grass snake. On the basis of their staining properties the dark green-coloured adrenaline-containing cells could easily be distinguished from the light, yellow-coloured, rough-granulated noradrenaline-containing ones.

In agreement with this histochemical picture, the electron microscope also revealed that the adrenaline- and noradrenaline-containing cells had light cytoplasm, and that their granules differed in size. These cells occurred either in isolation or in groups. The small granules, which, on the basis of their histochemical staining properties were proved to contain adrenaline, were of 500—2,000 Å size, and the larger, noradrenaline-containing granules of 1,500—3,500 Å size.

As a remarkable phenomenon, the large precursor granules among the catecholamine granules (Fig. 5) should be mentioned. These so-called "precursor granules" can be found in large number also in the adrenal medulla of the frog and the dog (unpublished data). In these granules we have often observed small masses, very similar to the adrenaline and noradrenaline granules, which — under certain conditions — seemed to be extruded into the cytoplasm. These large granules differ in size, density and structure from the "spherical bodies" described by ERÄNKÖ [6] in the rat, and, in our opinion, they represent a phase of catecholamine synthesis (dopamine?). We have also met these "spherical bodies" in the adrenal medulla of several experimental animals, such as the frog, grass snake, domestic fowl, rat, mouse, dog, etc., however, we consider them multivesicular bodies.

Since between the adrenaline- and noradrenaline-containing cells there is no other difference than the size of the granules in them, the question arises whether this is a satisfactory basis for the identification of a cell type.

Considering that structurally the two hormones are very similar, and that up to methylation their synthesis presumably proceeds in the same way, the presence or absence of the methylating enzyme may decide whether a particular cell will produce only noradrenaline, or both noradrenaline and adrenaline. On the other hand, the presence or absence of an enzyme is not necessarily associated with significant cell morphological differences.

Assessment of the so-called "dark cells" constitutes yet another problem. In our opinion the simultaneous occurrence of large and small granules in the same cell may be interpreted as follows: Some cells continuously contain those enzymes which are needed for the synthesis of adrenaline in the course of catecholamine synthesis. In these cells noradrenaline is only a transitory product. During activation of the methylating enzyme, the noradrenaline-containing granules may gradually transform into small granules containing adrenaline, with the result that at the same time both large and small granules will be present producing the dark cell type of transitory character (Figs. 4, 5). However, after completion of the methylating processes, only the adrenaline cells, containing small granules remain (Fig. 3).

On the basis of YATES'[17] and our own observations, the noradrenaline-producing capacity of the so-called "Nebenzellen" cannot be accepted without reservation, because when a homogenate of the adrenal medulla of the dog was sedimented with 3,000 g/30 min., it was found that 80% of the total catecholamine-content sedimented from the granules was noradrenaline (unpublished data). Thus the noradrenaline character of the large granules was proved both by sedimentation and biochemical methods. At the same time the "osmiophilic bodies" in the "Nebenzellen" described by WETZSTEIN differ substantially from the typical catecholamine granules. For sedimentation of the catecholamine-containing granules HAGEN and BARNETT [9] used 24,000 g/30 min. The gravitational power (3,000g/30min.) employed by us was, however, only sufficient for sedimentation of the larger ones of the noradrenaline granules.

As we have succeeded in establishing the place of production and storage of adrenaline and that of noradrenaline also with submicroscopic methods, there is hope that the complicated question of the selective secretion of adrenaline and noradrenaline may be approached also from the point of view of comparative morphology.

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HISTOCHEMISCHE UND ELEKTRONENMIKROSKOPISCHE UNTERSUCHUNGEN AM NEBENNIERENMARK DER WASSERSCHLANGE *(NATRIX NATRIX)*

Über die Struktur des Nebennierenmarkes der Wasserschlange gibt es weder in der optischen, noch in der elektronenmikroskopischen Literatur Angaben. In der vorliegenden Arbeit haben die Verfasser mit histochemischen und elektronmikroskopischen Methoden im Nebennierenmark der Wasserschlange drei Zelltypen nachgewiesen. Charakteristisch für den 1. Typ (»Noradrenalinzellen«) sind die »großen Katecholaminkörner«, mit einem Durchmesser von etwa 2500 Å, die Noradrenalin enthalten. Der 2. Zelltyp (»Adrenalinzellen«) ist durch die Anwesenheit der sogenannten »kleinen Katecholaminkörner« gekennzeichnet, welche einen Durchmesser von etwa 1250 Å haben und Adrenalin enthalten. Beide Zelltypen besitzen helles Zytoplasma, arm an Zellorganellen. Im 3. Typ, in den sogenannten »dunklen Zellen«, sind beide Arten der Katecholaminkörner (»kleine« und »große«) zu finden. Außerdem wurden in den »dunklen Zellen« 0.5—1.5 μ große Dopamin Precursorkörner, Golgi-Apparate, multivesiculäre Körper, wenige Mitochondrien, viele Palade-Körner und vesiculäres Ergastoplasma beobachtet.

ГИСТОХИМИЧЕСКОЕ И ЭЛЕКТРОННОМИКРОСКОПИЧЕСКОЕ ИССЛЕДОВАНИЕ НАДПОЧЕЧНИКА ВОДЯНОГО УЖА *(NATRIX NATRIX)*

В оптической и электронномикроскопической литературе о вопросах, связанных со структурой мозга надпочечника *Natrix natrix* нет никаких данных. Настоящая работа, использующая гистохимические и электронномикроскопические методы, подтвердила, что в мозге надпочечника *Natrix natrix* можно различать три типа клеток. Для первого типа клеток (норадреналиновые клетки) характерно, что в них можно видеть большие катехоламиновые гранулы диаметром около 2500 Å. Эти клетки содержат норадреналин. Второй тип клеток можно определить на основе наличия в них маленьких катехоламиновых гранул, диаметр которых около 1250 Å. Эти гранулы содержат адреналин. Оба типа клеток имеют светлую цитоплазму и клеточные компоненты в них немногочисленны *Natrix natrix*.

Кроме описанных двух типов клеток, есть еще и третий тип, так называемые «темные клетки», в мозге надпочечника *Natrix natrix* где можно видеть оба типа катехоламиновых гранул (большие и маленькие). Кроме этих гранул в этом типе клеток можно найти допаминовые гранулы, содержащие прекурзор (в диаметре 0,5—1,5 μ), аппараты Голджи, мультивезикулярные тельца, небольшое число митохондриев, в большом количестве гранулы Паладе, и эргастоплазму везикулярного типа.

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Acta Biol. Hung., **15** (3), 285—298 (1965)

ELECTRON MICROSCOPIC STUDY OF ADRENALINE AND NORADRENALINE SECRETION OF THE ADRENAL MEDULLA

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(Received April 5, 1964)

Synopsis

We studied adrenaline and noradrenaline secretion, elicited by insulin load, with physiological and electron microscopical methods in the adrenal medulla of the white laboratory rat and the dog. Under the effect of 10 IU/kg insulin injected subcutaneously into the rat we measured a 93.4% decrease of adrenaline content 6 hours after administration with a simultaneous 33% increase in noradrenaline content. In the electron microscopic picture, parallel with the decrease of adrenaline, we observed a 40% decrease in "small granules" (about 1,000 Å); at the same time the number of "large granules" (2,000 Å) increased to 60%. Intravenously administered 4 IU/kg insulin gave a similar result in the dog, but the quantitative results were less definite. Insulin-treated medullary cells show characteristic submicroscopic changes as compared with the normal picture. Thus the cytoplasm is of the "light" type, highly vacuolized and contains only a small number of secretory granules. Golgi apparatuses are observable in great number and extensive forms and are in close connection with the secretory granules, mitochondria and ergastoplasm. In the adrenal medullary cells of the normal dog, beside catecholamine granules we identified with submicroscopic and chemical methods dopamine granules of precursory character and 0.5—1.5 μ in size.

Introduction

Concerning the ultracytological analysis of the secretory activity of the adrenal medulla, few data of literature are known. Lever [12] was the first to examine the submicroscopic structure of the adrenal medulla in normal and denervated rats, and rats exposed to the effect of cold. Following exposure to cold, Lever noticed the numerical increase of light cells and linked this phenomenon with the evacuation of secretory granules. Wetzstein [17] performed electron microspic examinations on the adrenal medulla of mice, guinea pigs and cats and found that, compared with the normal, the electron absorption of catecholamine granules significantly decreases under the effect of insulin. He attributed this fact to the consequences of active adrenaline-mobilization. Wetzstein claims the merit of first connecting electron microscopic examination with results obtained by histochemical and biochemical methods. This comparison showed good agreement with the quantitative changes of adrenaline. These examinations as well as the results in granule-fractionizing of Hillarp [10] have undoubtedly proved that the adrenaline and nor-

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adrenaline content of the adrenal medulla is present in the cytoplasm in the granular form. From the ultracytological viewpoint, however, it remained to be clarified whether a definitely distinguishable submicroscopical representation of these two kinds of hormones existed or not. YATES [18] was the first to give a reassuring answer to this question. Starting from the histochemical data of ERÄNKÖ [7] he separated with a phasecontrast microscopic and electron microscopic method adrenaline cells containing small granules (700—1,000 Å), and noradrenaline cells producing large granules (1,000—3,500 Å) in the adrenal medulla of the gold hamster. These concepts of YATES were supported by similar results obtained by WASSERMANN [16] in snakes and BENEDECZKY and co-workers [2] in grass snakes. The reliable identification of these two kinds of granules is essentially important for understanding the mechanism of selective secretion of adrenaline and/or noradrenaline. Knowing the selective adrenaline mobilizing effect of insulin, insulin load was applied to dogs and rats to identify — relying on the electron microscopic analysis of the mobilization of catecholamine granules — adrenaline resp. noradrenaline granules by their different size and evacuation. In addition we endeavoured to study the electron microscopic structure of medullary cells, whose hyperactive state was elicited by insulin loading and the ultrastructural changes of cell components (Golgi apparatus, ergastoplasm, mitochondria) involved in the secretory processes.

Material and method

In our experiments we used laboratory white rats and dogs. On the day before insulin load was applied we removed unilaterally the adrenals of the rats. After weighing the adrenals were suspended in 5% trichloroacetic acid. After filtering the extract was shaken with aether to remove trichloroacetic acid. The quantity of wet-phase catecholamine—divided into two equal parts — was oxidized on pH 4, pH 6 values with iodine oxidation into adreno-, resp. noradrenochrome. The coloured product has been photometrized in a Pulfrich photometer on 529 mμ [15]. The adrenals of 22 rats were used for purposes of normal control. Insulin load was applied on the first postoperative day. 10 IU insulin/kg body weight was injected subcutaneously into each animal. To prevent insulin shock we gave intraperitoneally 1—2 ml 5% dextrose per animal. Six hours after the administration of insulin the rats were decapitated and their adrenals cut in two equal parts. One half was used for chemical determination while the other for electron microscopic embedding. In the case of dogs, bilateral adrenals of 5 animals were used for the determination of adrenaline and noradrenaline values. Insulin load was applied to five animals and 4 IU insulin/kg body weight given intravenously. One and half hour after the administration of insulin we killed the animals by intracardial administration of ether. Simultaneously with the chemical determination, we took samples of the adrenal medulla of each animal for electron microscopic purposes. For the preparation of electron microscopic material we prefixed for 48 hours medullary parts of 1 mm^3 with a method developed by us [1] in 3.5% formalin (pH 7.6). Subsequently fixation was carried out for 1 hour, according to PALADE [14]. Embedding was performed with Mercer's [13] method in araldite, sectioning with LKB Ultrotome. Section thickness: 300—500 Å. Contrasting was performed with lead, according to KARNOVSKY [11]. Electron microscopic pictures were made with an ELMI D_2 (Zeiss, Jena) apparatus. The diameters of the catecholamine granules were measured on 13 × 18 cm figures with a 16,000 × magnification. We ranged the granules of 2 mm (about 1,000 Å) and of smaller diameter into the group of "small granules". Those having a diameter above 2 mm (1,500—3,500 Å) formed the group of "large granules".

Results

We found the mean value of adrenaline content of the normal rat adrenal medulla to be 1034 μg/g (350—1800 μg/g), the noradrenaline content, however, to be 480 μg/g (210—1700 μg/g). Under the effect of 10 IU/kg body weight insulin injected subcutaneously, the adrenaline content of the adrenals decreased by 93.4%* after 6 hours. On the other hand, a 33% increase was experienced in the noradrenaline content** (Fig. 1).

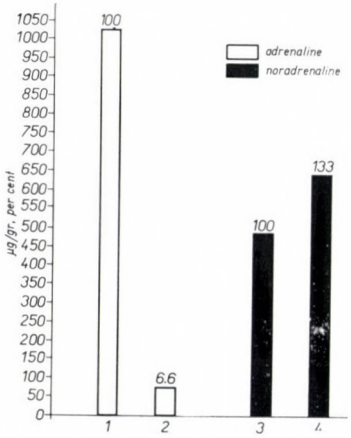

Fig. 1. The catecholamine content of the rat's adrenal in normal state, and 6 hours after the subcutaneous administration of 10 IU/kg insulin. 1. The adrenaline content of the contralateral control. 2. Adrenaline content 6 hours after the administration of insulin. 3. The noradrenaline content of the contralateral control. 4. Noradrenaline content 6 hours after the administration of insulin

For evaluating the ultrastructural changes caused by insulin-effected adrenaline mobilization, first we studied cells of the normal adrenal medulla, and found that the "dark type" of cytoplasm and its rich pattern in cell organelles is characteristic of the electron microscopic structure of the normal adrenal medulla. The membrane of the plasma shows mostly a double arrangement, in some places, however, it is blurred. The most conspicuous structures of the cytoplasm are the catecholamine granules of great density, bordered by a membrane, or membraneless, having mostly rounded profiles, which in the normal cells are observable generally in even distribution. The secretory granules are in some instances in immediate contact with the cytoplasm, whereas in other cases, they have a so-called "light halo".

* Mean value: 68 μg/g, extreme values: 50—180 μg/g.
** Mean value: 650 μg/g, extreme values: 500—1800 μg/g.

The ergastoplasm appears generally in a "vesicular" form, the lamellar type can be found but rarely. The membranes of the ergastoplasmic cistern contain in many instances only very few granules and sometimes seem to be entirely devoid of them. In some cases so-called "intracisternal catecholamine granule" is present in the light lumen of the cistern. Besides, numerous mitochondria and Palade granules of diffuse distribution are found in the cytoplasm. The Golgi apparatus, however, is present mostly but in the light-type cells.

The size of the catecholamine granules vary on a wide range even within the same cell. Beside granules with very small diameter, of about 500 Å, granules of 1,000 Å dominate, but those of 2,000 Å are also represented in significant numbers. Of 685 counted and measured granules, 81% belonged to the group of "small granules" and 19% to that of "large granules" (Fig. 2).

As a result of insulin-effected adrenaline mobilization, significant changes took place in the cytoplasm. Among these the most striking were the "light" parts of the cytoplasm, totally free from granules, the empty membranes of the evacuated granules, and the presence of large cytoplasmic vacuoles. When measuring the diameters of residual granules in the medullary cells of insulin-treated animals, we found that, besides the relatively substantial increase of "large granules", the numerical ratio of the so-called "small granules" (500—1,000 Å) was considerably reduced as compared with the normal. Of 325 granules, 60% belonged to the large granules and 40% to the small ones. Comparing the trend in the change of biochemical data with the change in the intracellular ratio of granules of various size, it can be ascertained that a parallelism exists between adrenaline and the numerical decrease of small granules, as well as between noradrenaline content and the numerical increase of the large granules (Fig. 3).

Golgi apparatuses can be observed in great numbers and extended forms in insulin-treated medullary cells. In the Golgi apparatus shown in Fig. 3, numerous electron-dense granules may be observed in addition to the likewise great number of Golgi vesicules. The numerous, highly developed lamellae, vacuoles and vesicles, the secretory granules gathering in the Golgi-region, and the very close morphological connection of Golgi-mitochondrium-ergastoplasm evidence the effective function of the Golgi apparatus.

During our further experiments, we have found the cell-type described in rats also in the normal dog. Here the cytoplasm is of the dark type, very rich in Palade granules (Fig. 4).

The majority of catecholamine granules is confined by membranes, some of them have a light halo, while others have neither haloes nor membranes. In the adrenal medullary cells of the normal dog the ratio of the "large granules", as related to the rat, is higher and, on the average, reaches 35%. In this instance the number of measured granules was 812. It should be noted that

according to literary data noradrenaline content in the dog adrenal reaches as much as 30—50% of the total catecholamine quantity [5]. During our examinations we found the adrenaline content of the dog adrenal to be 850 μg/g, as mean value. (Extreme values: 500—900 μg/g.) The average value of noradrenaline content was 430 μg/g. (Extreme values: 400—600 μg/g). For further observation of granule mobilization, we applied insulin load also to dogs (4 IU/kg, i.v.) (Fig. 6).

Beside the 37% decrease in adrenaline (mean value 500 μg/g; extreme values 480—850 μg/g) the noradrenaline content itself remained essentially unchanged (mean value 410 μg/g, extreme values: 400—560 μg/g). Comparing this value with that obtained in the rat (93.4% after 6 hours) we ascertained that during one and a half hour a relatively great quantity of adrenaline was mobilized, on the other hand, no increase in noradrenaline was observed.

In the electron microscopic pictures of the adrenal medullary cells, the changes described in the rat were manifest only to a lesser extent (Figs. 5 and 7).

In some cases the cytoplasm is of the "light" type, highly vacuolized in many places; elsewhere, however, it retained its "dark" character. In conformity with the 37% decrease in adrenaline we found cells (Fig. 5) in which adrenaline mobilization was accompanied by a significant numerical decrease of the granules, but we have observed also cells in which the characteristic ultrastructural changes following evacuation were not significant (Fig. 7, "dark" cell).

Based on the diametric determination of the granules, the "large granule" fraction was 54% in 570 catecholamine granules. The ratio of the small granules decreased from 65 to 46%. Since, according to chemical data, the noradrenaline content showed no significant change under the effect of insulin treatment, increase in the percentual value of the large granules is only a relative numerical increase, since due to adrenaline secretion, the distribution of the two kinds of granules shifted in favour of the non-secreting large granules.

Beside the above-described cell type in the adrenals of the normal dog we have observed also cells in which, apart from typical catecholamine granules much larger, 0.5—1.5 μ sized membrane-bordered granules are also present which have a finely granulated internal structure (Fig. 8).

So far, no mention has been made in electron microscopic literature of the adrenal medulla of this type of granules. One of their characteristic features is their varying electron-absorption, but they are always lighter than the typical catecholamine granules. It is frequently observable that in one of their poles a small granule of great density is present, reminding of catecholamine granules. We have also observed in several instances that catecholamine granules can be seen adjacent to the loosened discontinuous membrane of

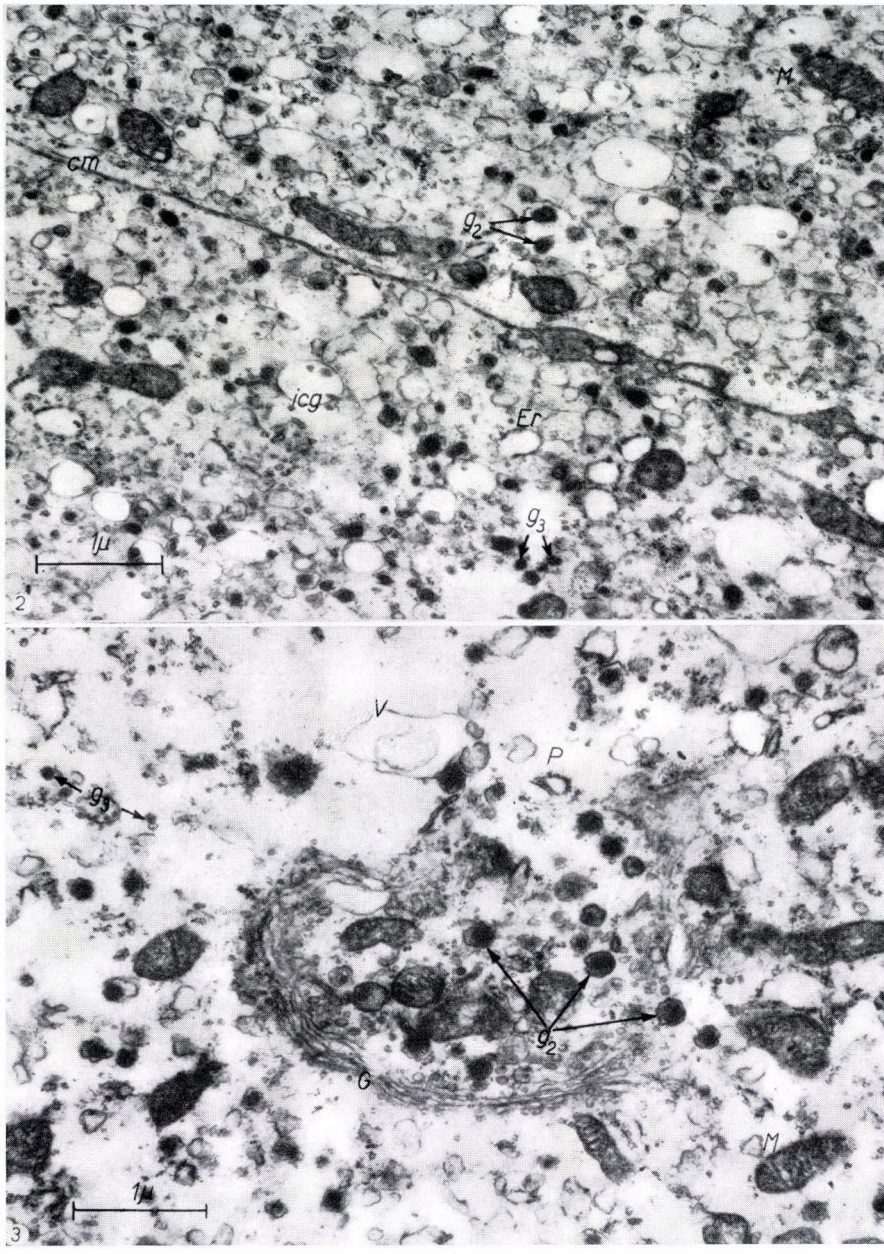

Fig. 2. Normal adrenal medullary cell in rat. The "dark"-type cytoplasm contains numerous secretory granules (g_2, g_3) in even distribution. Ergastoplasm (Er) is present in a vesicular form. Intracisternal granules (icg) are observable in several places. The mitochondria (M) have an elongated form, of dark character. A great number of Palade granules (P) can be seen in the cytoplasm. × 18,000

N: nucleus
Ne: nuclear membrane
G: Golgi apparatus
Er: ergastoplasm

these granules giving the impression that they are in the phase of extrusion from the large granule (Fig. 8).

Discussion

Though several authors have studied the submicroscopic organization of the adrenal medulla [2, 4, 8, 12, 16, 17, 18], until quite recently no definite data of universal validity could be found regarding the ultrastructure of this organ. LEVER [12] was the first to study in 1955 the submicroscopic structure of the normal and denervated adrenal medulla of the rat, and that exposed to the effect of cold, and in the course of investigations drew several noteworthy conclusions. Those observations which refer to the form, size, density and internal structure of secretory granules are essentially valid even today. It became evident for LEVER that the size of secretory granules within the normal cells changed over a wide range. He did not attach, however, particular importance to this observation and ascribed this change in size to adrenaline synthesis and to the momentary state of secretion respectively. Only much later, in 1962, did YATES and co-workers [18] come to the conclusion that granular size can be principal determinant of a cell-type. With histochemical and electron microscopical methods they isolated in the gold hamster two cell types containing granules of different size. The peripheral cells, containing mainly large granules (on the average, 2,000 Å), proved to be histochemically noradrenaline-producing cell varieties, while in the cells containing small granules (1,000 Å), they succeeded in tracing adrenaline. Electron microscopically and histochemically we succeeded in demonstrating these two cell-types and two kinds of granules in the grass snake [2]. All these observations represent a fundamentally new viewpoint regarding adrenaline and noradrenaline secretion of the adrenal medulla. Namely, in the following we cannot speak of catecholamine granules in general as does WETZSTEIN [17], but of granules containing adrenaline and/or noradrenaline (at least in case of the species mentioned). It seems also probable that the "osmiophilic corpuscles" of the "Nebenzellen" described by WETZSTEIN are not identical with the noradrenaline "ranules. Namely HAGEN and BARNETT [9], as well as BENEDECZKY [3], isolat-

cm: cell membrane
M: mitochondria
g_1: dopamine granule
g_2: noradrenaline granule
g_3: adrenaline granule
V: vacuole
P: Palade granule
bm: basal membrane
icg: intracisternal granule

Fig. 3. Adrenal medullary cell, insulin-treated rat, 6 hours after the administration of insulin. The cytoplasm is of the "light type", its small-granule (g_3) content is significatly reduced. The great majority of granules present can be ranged into the large granule (g_2) group. The Golgi apparatus (G) is composed of well-developed lamellar and vesicular components. Several "large granules" (g_2), mitochondria (M) and Palade granules (P) are observable in the Golgi-zone. A significant number of empty vacuoles (V) can be found in the cytoplasm. × 18,000

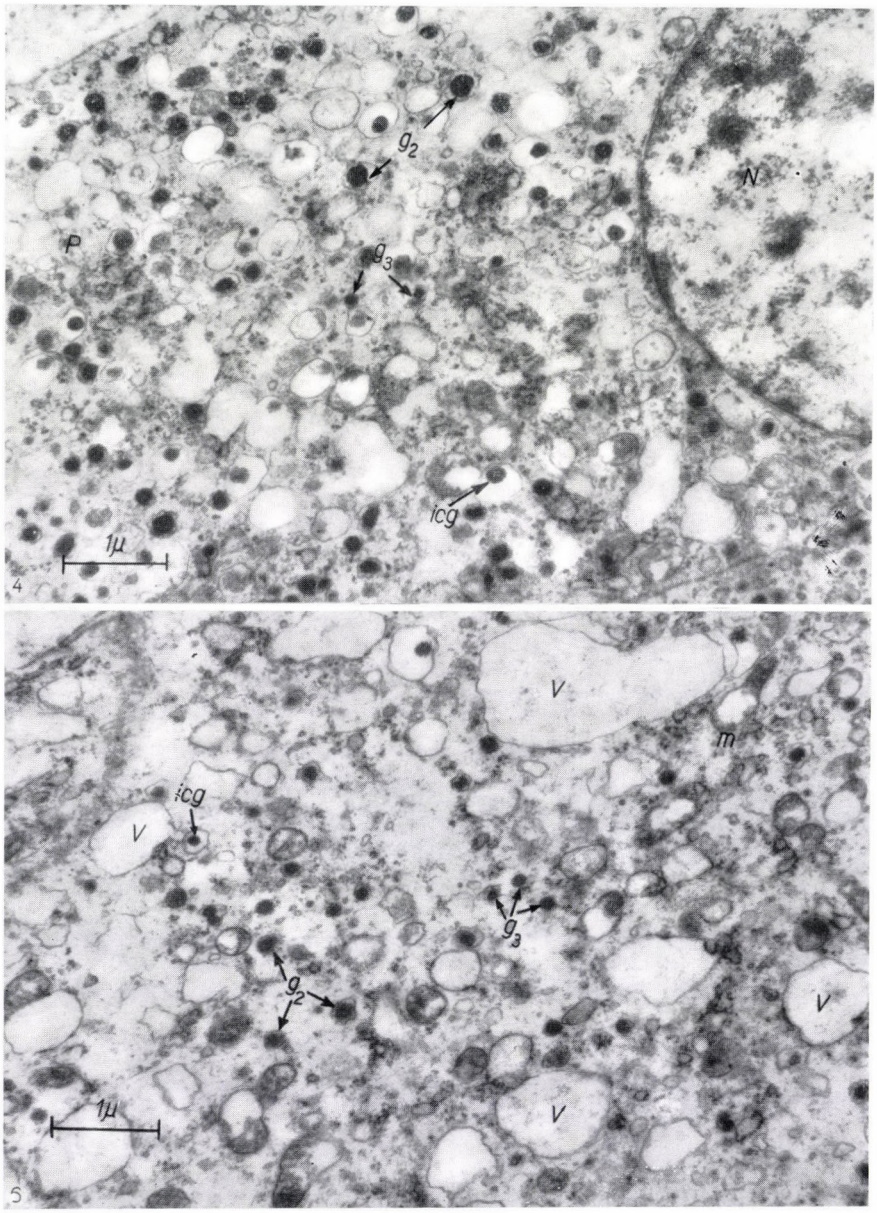

Fig. 4. Normal adrenal medullary cell of dog. Catecholamine granules (g_2, g_3) are situated in the dark-type cytoplasm either freely surrounded by a membrane or in cisterns with a smooth surface (icg). The secretory granules show a significant difference in form and size. Palade granules (P) are present in great quantities rallied into zones. × 15,000

Fig. 5. Portion of adrenal medulla, dog, 1.5 hours after insulin administration. The cytoplasm is lighter than the normal and contains several vacuoles (V). The numerical decresae of secretory granules is especially manifest in case of the intracisternal granules (icg). Among the catecholamine granules present, those with 1,500—2,000 Å diameter (g_2) dominate as against the small ones (g_3); × 15,000

ed by ultracentrifugation two granule fractions from the homogenized adrenal medulla of the cow and dog respectively and found the greater part of the first sedimentation fraction of greater specific gravity (in dogs about 70%) to be noradrenaline. Based on these data, further on diameter determinations of granules made in the rat, dog and snake, we are of the opinion that in case of lower objects (frog, snake) two kinds of cells, one producing adrenaline, the other noradrenaline, function simultaneously.

As opposed to YATES [18] and to our earlier results [2], we did not succeed in observing cells of purely small granules in rats and dogs. The catecholamine

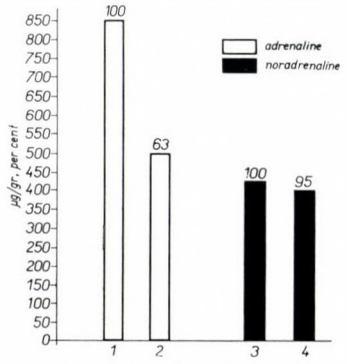

Fig. 6. Catecholamine content of the dog's adrenal in the normal state and one-and-half hour after iv. administration of 4 IU/kg insulin. 1. Adrenaline content of the control. 2. Adrenaline content one-and-half hour after insulin administration. 3. Noradrenaline content of the control. 4. Noradrenaline content one-and-half hour after insulin administration

granules of normal medullary cells in rats and dogs show great differences in size within each cell. In the rat, e.g. 81% of all granules have a diameter of 1,000 Å or less, (so-called "small granules"), while 19% are of 1,500—2,000 Å ("large granules"). This proportion is in good agreement with the chemically determined normal adrenaline and/or noradrenaline content (which, according to our results, is 1,034 µg/g adrenaline resp. 480 µg/g noradrenaline) if we take into consideration that the "volume of the large granules" with their diameter almost twice as large, represents a much greater amount of noradrenaline.

In dogs and rats, in which only adrenaline is mobilized selectively by insulin, it can be stated that in all probability the remaining granules of 2,000 Å diameter contain noradrenaline. This presumption is in full harmony with biochemical data, according to which the quantity of adrenaline decreases by 93.4% under the effect of insulin load, beside the 30% increase in noradrenaline content. In the insulin-treated medullary cells the quantitative adrenaline decrease was followed by the numerical decrease of the "small granules". While in the case of the normal rat 81% of the total granules were small granules, in the medullary cells of the insulin-treated animals the share of the

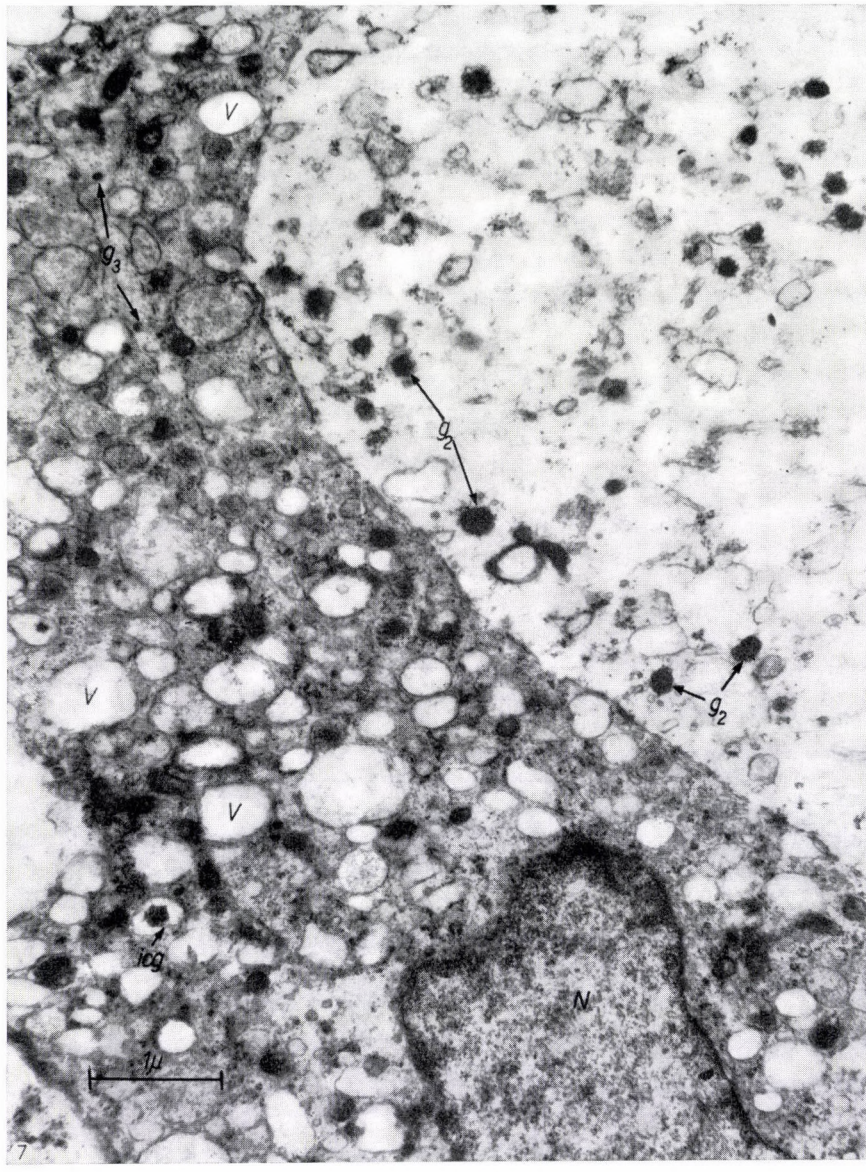

Fig. 7. Same as Fig. 5. It is well observable in the light cell that the cytoplasm is almost entirely free of granules, and those present belong to the large granules (g_2). The neighbouring dark cell retained its electron-dispersing capacity, although it contains at the same time, several empty vacuoles (V). Also small granules (g_3) occur in the dark cell. × 18,000

"small granules" sank to 40%. The 33% increase in noradrenaline content, following the insulin load was, on the other hand, accompanied by a rise to 60% in the number of large granules. Insulin load applied to the dog — as discussed when surveying the results — has shown a similar effect, but this

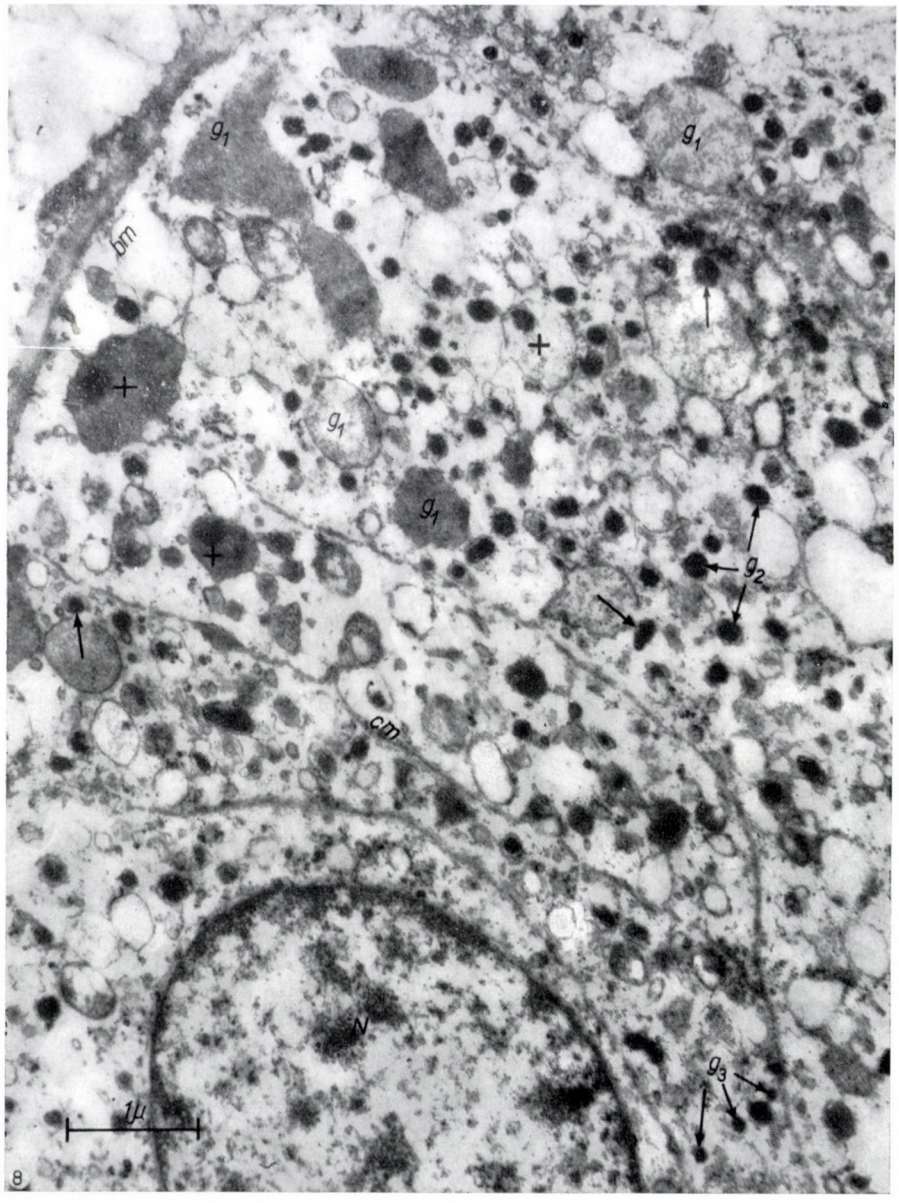

Fig. 8. Normal adrenal medullary cells of dog with 0.5—1.5 μ dopamine granules (g_1) and catecholamine granules (g_2, g_3). The bordering membrane, finely granulated internal structure and the great difference in their electron-dispersing character is well observable even within the same cell. Within the dopamine granule marked with a cross (+) a structure reminding of the catecholamine granule is present, while in the place marked with an arrow (→) the membrane discontinuity is discernible and next to it the secretory granule of great density and catecholamine character, presumably in the phase of shooting out, or in the period immediately following it. × 18,000

change has not reached the quantitative values measured in rats. This quantitative difference is, however, easy to understand by the fact that the load was applied for only one-and-half hour and the quantity of the insulin injected was considerably smaller (4 IU/kg) than in case of the rat. Based upon all these observations we presume that "small granules" of adrenaline content, and "large granules" of noradrenaline content coexist within each individual cell in the adrenal medullary cells of the rat and dog.

We find the presence of 0.5—1.5 μ size granules in the medullary cells of the normal dog, and their role in respect to catecholamine synthesis, highly interesting. None of the researchers engaged in the investigation of the adrenal have reported on granules of such size, form and density. ERÄNKÖ [8] has, however, mentioned so-called "spherical bodies" when dealing with one cell-type of the rat adrenal, but has offered no concept whatsoever regarding their origin or role. It is surprising that neither LEVER [12] nor other authors [16, 17, 18] should have found these structures either in normal or in insulin treated medullary cells of rats. On the other hand, the 0.5—1.5 μ (large) granules observed by us in dogs occur in lower or higher numbers also in the rat. We believe it important to note that we did not succeed in finding these structures when using methacrylic embedding. Following embedding in araldite, however, they could be well recognized and identified in great numbers. Since the majority of works published on the adrenal medulla are based on methacrylic embedding, we infer that it is due to the harmful effect of this embedding substance that other authors were unable to demonstrate these specific precursor granules. The fact that within these specific granules very frequently a smaller granule (1,000—2,000 Å) of great electron-absorption is present, leads us to the conclusion that we are confronted with a precursor granule of adrenaline and/or noradrenaline which, as a result of our recent examinations [3], was found chemically to be dopamine. The discontinuities to be observed on the membranes of these granules, which might serve as shooting spots for the ripe small granule are highly thought-provoking.

From among the cell organelles of the adrenal medulla, we know especially little about the Golgi apparatus. WETZSTEIN [17] demonstrated it only in "Nebenzellen". DE ROBERTIS [6] on the other hand finds the Golgi-nature of this organellum, described in the "Nebenzellen" questionable and regards it as a degenerated mitochondrial growth observable in the zona fasciculata. Though LEVER [12] published an illustration in which the Golgi apparatus is marked, it reveals very little of the fine structure of the Golgi apparatus. We succeeded in finding the Golgi apparatus [2] in cells of normal condition in the dog, rat, frog and also in the snake, and according to the classical definition it is composed of twin lamellae, a great number of vesicles, and Golgi vacuoles. 5—6 hours after the insulin load when — according to biochemical data — a considerable part of adrenaline has evacuated from the adrenal, we

succeeded in observing the Golgi apparatus in rats and dogs in the initial phase of resynthesis in a very highly developed form. In this hyperfunctional state the active Golgi apparatus is characterized by the following features:

(1) Several, highly developed twin lamellae with a great number of Golgi vesicles on their ends and marginal parts.

(2) Many Palade granules within the Golgi apparatus, several secretory granules of great density and varying size, highly electron-absorptive shortened mitochondria are observable.

(3) In certain spots, a direct morphological connection exists between ergastoplasm, mitochondrium and Golgi-lamellae, and also between Golgi vesicles and secretory granules.

Based on all these data we assume that also the Golgi apparatus is directly involved in the formation of the secretory granules which contain the two hormones of the adrenal medulla.

As to the mechanism of the formation of this secretory product, and the partial function by the Golgi apparatus in this process as a whole, no exact answer can be given yet at present.

The role of the lamellar, resp. vesicular form of the ergastoplasm in the preparatory, synthesizing, storing and evacuating processes of the secretory product remains an open question. A remarkable fact is that in the snake the large precursor granules, and in the dog also the catecholamine granules, are often surrounded with membranes of the ergastoplasmatic cistern, thus certain "granulo-genetic" processes take place in the cisterne of the ergastoplasm.

Our present observations are only of informative character and in order to obtain further adequate information, manifold experiments and their evaluations are needed.

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ELEKTRONENMIKROSKOPISCHE UNTERSUCHUNG DER ADRENALIN- UND NORADRENALINSEKRETION DES NEBENNIERENMARKES

Die durch Insulinbelastung ausgelöste Adrenalin- und Noradrenalinsekretion im Nebennierenmark von weißen Ratten und Hunden wurde physiologisch und elektronenmikroskopisch untersucht. Bei Ratten verminderte sich der Adrenalingehalt nach Einwirkung von 10 IE/Kg subcutan verabreichten Insulins nach 6 Stunden um 93,4%, der Noradrenalingehalt nahm gleichzeitig um 33% zu. In den elektronenmikroskopischen Aufnahmen beobachteten wir eine mit der Abnahme des Adrenalins parallel verlaufende 40%ige Verminderung der kleinen Granula (cca 1000 Å); gleichzeitig stieg die Zahl der großen Granula (cca 2000 Å) auf 60%, entsprechend dem Zuwachs des Noradrenalingehaltes. Ein ähnliches Resultat ergaben auch beim Hund intravenös verabreichte 4 IE/Kg Insulin, doch waren die quantitativen Veränderungen minder ausgeprägt. Verglichen mit den normalen Zellen, wiesen die mit Insulin behandelten Markzellen charakteristische Veränderungen auf; so z.B. das Zytoplasma vom »hellen« Typ, das stark vacuolisiert ist und nur wenige Sekretionsgranula enthält. Der Golgi-Apparat ist in großer Zahl und weit ausgebreiteter Form vorhanden und eng verbunden mit den Sekretionsgranula, den Mitochondrien und dem Ergastoplasma. Durch submikroskopische und chemische Methoden wurden in den Nebennierenmarkzellen des normalen Hundes, neben den Katecholamingranula, auch Dopamingranula von 0,5—1,5 μ Größe und Praecursor-Natur identifiziert.

ЭЛЕКТРОННОМИКРОСКОПИЧЕСКОЕ ИЗУЧЕНИЕ СЕКРЕЦИИ АДРЕНАЛИНА И НОРАДРЕНАЛИНА НАДПОЧЕЧНИКОВ

Секреция адреналина и норадреналина, вызванная введением инсулина была изучена в надпочечниках белых крыс и собак физиологическими и электронномикроскопическими методами. У крыс, в ответ на подкожно данный инсулин (10 E./kg), после 6 часов наблюдалось 93,4-процентное уменьшение адреналина, зато содержание норадреналина увеличилось на 83 процента. На электронномикроскопических снимках, параллельно с уменьшением адреналина, уменьшилось и число «мельких гранул» на 40% (в среднем 1000 Å), в то время как число «больших гранул» (2000 Å) увеличивалось на 60 процентов, параллельно с увеличением содержания норадреналина. У собак после подкожного введения инсулина (4 E/kg) полученные данные были те же самые качественно, но количественно менее выражены. После введения инсулина, клетки мозгового слоя надпочечника показывали характерные субмикроскопические изменения по сравнению с нормальными. Так, например, цитоплазма светлого типа, сильно вакуолизированная, содержит в себе мало секреционных гранул. Аппарат Голджи наблюдается в больших количествах, в расширенной форме и находится в тесной связи с секреционными гранулами, митохондриями и с эргастоплазмой. В мозговых клетках надпочечника нормальной собаки субмикроскопическими и биохимическими методами обнаружены также гранулы содержащие допамин, типа прекурзора, диаметром около 0,5—1,5 μ.

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Acta Biol. Hung., **15** (3), 299—310 (1965)

OXYGEN LEVEL AS A SPECIFIC REGULATOR IN THE RHYTHMIC ACTIVITY OF FRESH-WATER MUSSEL (ANODONTA CYGNEA L.)

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(Received April 20, 1964)

Synopsis

Studies were performed to investigate the influence of O_2-deficiency on the periodic activity of *Anodonta cygnea*. It was established that the duration of continuous activity is reduced and the number of its alternations with periods of quiescence is considerably increased if the dissolved O_2-content of water decreases. This effect does not occur if the nerves are cut through around the gill and siphon. On the basis of the above results and previous findings demonstrating the susceptibility of periodic activity to substances influencing tissue respiration it is inferred that in the regulation of periodic activity metabolic processes occurring in the periphery, further — if natural conditions are considered — first of all the oxygen level of water and aerobe and anaerobe processes in the tissues being in direct contact with water are involved. In the course of studies recorded here, no significant differences were found in the lactic acid content of the various organs of animals being either well supplied with oxygen or living in water containing ten times less O_2, although the amount of consumed O_2/100 g living material expressed in the average of 24 hours was 1.11 mg/hour in the case of the former and 0.14 mg/hour in the case of the latter. Thus, it is inferred that in the regulation of the biological rhythm of *Anodonta* there are no periodically alternating external factors involved, but that it is the function of the free O_2-content of the surrounding water and of the aerobe-anaerobe respiratory processes taking place in the tissues being in direct contact with the environment.

Introduction

Periodicity which is generally occurring in living organisms manifests itself in different ways in the various species of bivalves. These experiments were performed first of all on marine lamellibranchiates. In the case of these animals rhythmic activity was observable in several physiological processes as feeding [12, 13], filtration activity [3, 14], O_2-consumption [6], motility [5, 10] and presumably in other processes. In the case of fresh-water mussels it was the movement of the valves, i.e. the periodicity in the activity of the adductors which attracted attention [1, 7, 21].

The periodic activity of marine bivalves is regulated decisively by the diurnal alternation of illumination and t^0, by ebb and tide and the rhythmic interchanges of direct environmental factors, i.e. it is the periodicity of these factors with which activity changes coincide.

Under laboratory conditions a certain degree of diurnal fluctuation could be demonstrated both in rhythmic activity and in the periodicity of activity of fresh-water mussel (*Anodonta cygnea* L.) [20]. Diurnal changes are, however,

not characteristic, their degree is not considerable and they may be demonstrated only by computations of averages from values obtained on rich experimental material. The increase in adductor activity in the morning is demonstrable only by statistical computations, otherwise the tonic contractions lasting for several hours (rest period) and similarly active condition consisting of frequent, rapid adductions and abductions are occurring with considerable regularity in the various periods of the day.

The fact that there is no close relationship between the rhythmic changes of environmental factors and this biological rhythm of fresh-water mussel raises the question whether or not the regulation of periodic activity is founded on factors other than those established in the case of the daily ebb and tide and other rhythms [4, 9].

In the course of his studies BARNES [1] observed that the periodic activity of *Anodonta* is not only independent of diurnal changes, but also of the food-, and O_2-level of the surrounding fluid as well as of the CO_2 and other metabolic products present. In contrast to these findings it has been shown previously [7, 15] that the periodic activity of *Anodonta* might be influenced by certain chemical substances, especially by KCl and SH-inhibitors. The number of rest periods occurring periodically within a unit of time is considerably increased by both KCl and SH-inhibitors, i.e. the single periods of continuous activity become considerably shorter and the duration of total activity per unit of time decreases considerably. These results show that the periodic activity of *Anodonta* is susceptible to influences produced by external factors. In the course of earlier studies it was established that it was the interference of the receptors located in the periphery through which these effects manifested themselves [17, 18, 19]. Studies on the possibility of eliminating the effect of KCl and SH-inhibitors suggest that both damaged and optimum aerobe respiratory conditions my play an important role in influencing periodic activity [16].

These facts, however, not only refute the statement, that periodic activity is independent of O_2-supply, but even lead to the assumption that from among physiological circumstances it is O_2-supply that is primarily important in the regulation of periodic activity.

In that case, however, not the regulating factor i.e. the oscillation of O_2-level would form the basis for the regulation of rhythm, but its lasting, constant values would by way of an inner mechanism influence periodic activity.

Studies presented here were performed to learn which way periodic activity of *Anodonta* is influenced by the O_2-level of the water surrounding the animal and to investigate the changes produced by hypoxygenic respiratory conditions in the activity of adductors and in the metabolic processes of the animal itself.

Method

For the studies 12—15 cm. long specimens of *Anodonta cygnea* were used, which were kept previously in an aquarium for several weeks. The activities were recorded with a special slow actograph writing in ink [21]. This apparatus made a continuous observation of several days possible. During investigation the animals were not fed and they were kept in separate vessels containing 2.5—3 litre water each.

At eight o'clock every morning samples were taken regularly from the water and their O_2-content was determined by Winkler's method. The O_2-content of the water was increased by water-circulation or by the aeration of the water. The quantity of dissolved O_2 was reduced by boiling the water, which was thereafter cooled in a hermetically closed bottle and poured into the tank containing the experimental animal.

In certain experimental runs paraffine oil was poured on the surface of the water to prevent further oxygen going into solution from the surrounding air either after the addition of boiled water of after the consumption of dissolved O_2 by the animal.

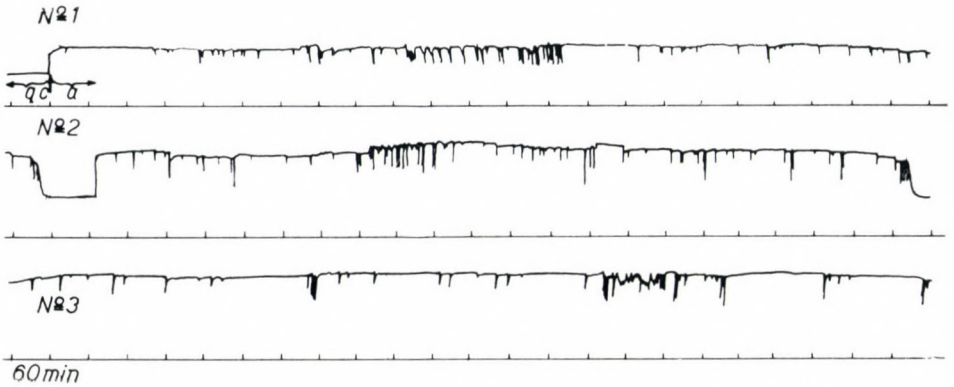

Fig. 1. The periodic activity of three fresh-water mussels under optimal environmental conditions. Time: 1 hour, "q" — period of quiescence, "c" — "change", "a" — period of activity

Lactic acid determinations in the tissues were made by the paraoxydiphenyl micromethod [24]. Lactic acid content was determined in the following organs: adductor muscle, gill, mantle and the photosensible region around the siphon of the mantle. For chemical analyses the organs from one side and only one half of the muscles were used. The organs from the other side and the other half of the muscles served for dry-matter determinations.

Results

Relationship between periodic activity and O_2-content of water

Animals in 12—15°C streaming water of high O_2-level are extraordinarily active. This implies that under such circumstances the animals are in active, open condition during 18—22 hours in a day. The duration of the periods of lasting closures amounts to at least 2—6 hours whithin 24 hours. The alternation of periods of activity and quiescence ("change") in these animals occurs at least once or twice daily. These results are illustrated in Fig. 1. It is seen that the duration of active periods is 23, 22 and 24 hours, and the number of changes is 1, 3, and 0 in animals No. 1, 2 and 3 respectively. The O_2-consumption of the experimental animals was considerably low under such experimental conditions (about 0.6—1.6 mg/100 g wet weight/hour at 16—18° C).

In the experiments five animals were subjected under similar conditions to parallel investigation. In one experimental run the conditions were changed in the subsequent days as follows:

in the first 24 hours optimal conditions were maintained by aeration and water circulation;

during the second 24 hours the exchange of water was still maintained but aeration was discontinued;

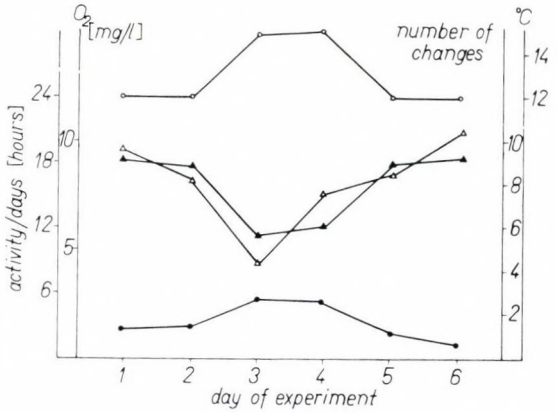

Fig. 2. The formation of periodic activity upon changes in O_2-level. Explanation in the text $-\triangle-\triangle-$ activity; $-\blacktriangle-\blacktriangle-$ O_2-content of water; $\bullet-\bullet-\bullet$ number of closures; $-O-O-$ temperature of water

during the third 24 hours also water-exchange was suspended;

the conditions in the fourth, fifth and sixth days were the same as in the 3rd, 2nd and 1st day, respectively. Since the free O_2-content of water is influenced by changes in temperature, water-temperature was also recorded regularly. The results obtained are graphed in Fig. 2.

Figures summarizing the data on five animals show that there is a decrease in the duration of activity in the subsequent 24 hour periods parallel with the decrease in O_2-content of water. At an O_2-content of 9.24 mg/litre the daily average duration of activity was 19.2 hours, which fell to 8.7 hours with a decrease of O_2-content to 5.6 mg/litre.

After some time the alternation of periods of activity and quiscence increased, being doubled if extreme values are considered. With the rise of O_2-level the reciprocal results were obtained.

The temperature of water measured at eight o'clock happened to influence the changes in O_2-content during the experimental run most favourably. This, however, raises the question whether perhaps t° was the single factor that influenced periodic activity independent of O_2-level. This problem was solved by the second experimental run, in which the temperature was kept constant for several days.

In the course of the second experimental run not only the exchange of water and the aeration thereof was stopped after the first 24 hours, but a layer of neutral paraffine oil was poured simultaneously on the surface of water to prevent further oxygen absorption of the water for the replacement of O_2 consumed by the animals.

Water samples were taken during three subsequent days at 24 hour intervals for O_2-determination. The results of these measurements are shown

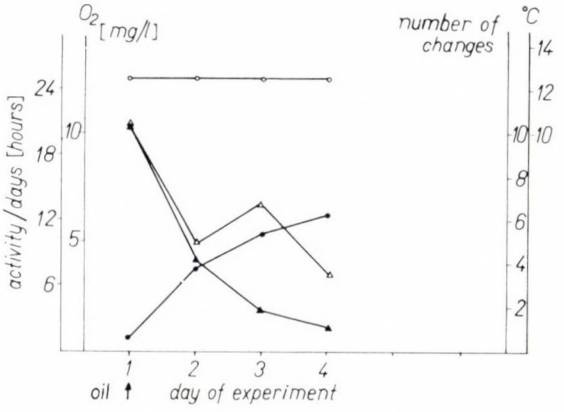

Fig. 3. The formation of periodic activity under hypoxygenic conditions. Explanation in the text.
$-\triangle-\triangle-$ activity; $-\blacktriangle-\blacktriangle-$ O_2-content of water; $\bullet-\bullet-\bullet$ number of closures; $-O-O-$ water temperature

in Fig. 3. It appears from the figure that the decrease in activity observed in the first 24 hours is followed by a simultaneous decrease of dissolved O_2-content, and there is a considerable increase in the number of diurnal "changes". In this experimental run the temperature of water measured every morning was constant.

In view of the above results it is considered highly probable that under the given conditions harmful metabolic products accumulate inside the closed liquid area and that their presence might perhaps be responsible for the characteristic changes in periodic activity. In order to elucidate this problem $^1/_2$—1 litre fresh water rich in O_2 was added into the vessel containing the animal. On another occasion 200—300 cm^3 water was scooped out from each of the containers saturated by air-bubbling with O_2 and replaced thereafter under the oil layer. The results obtained were the same on each occasion: the duration of activity increased for shorter or longer periods depending on O_2-level (Fig. 4), irrespective of the fact that the harmful metabolic products were not removed and not diluted even to a minimum in the second case. The figures presented here illustrate experiments performed in non-aerated water at a temperature of 19° C. This explains the relatively low initial O_2-value.

These studies point to a decisive role of the quantity of dissolved O_2 in the regulation of the periodic activity of *Anodonta*.

Since the influences of KCl and SH-inhibitors on periodic activity could be eliminated by the denervation of siphon and gill [18, 19] experiments were launched to learn whether the earlier effect of O_2-deficiency still exists after the intersection of nn. palliales post. maiores and nn. branchiales. It was found that in that case O_2-deficiency did not induce changes in periodic activity (Fig. 5).

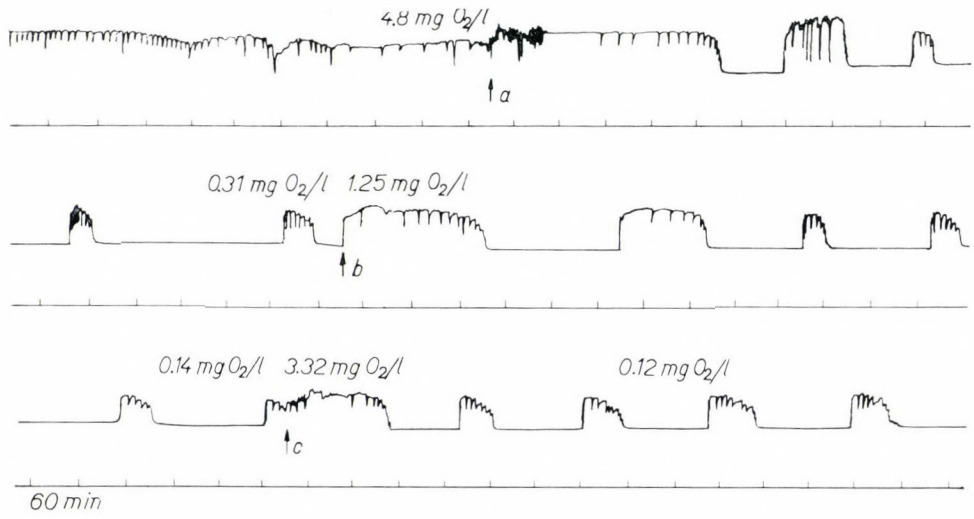

Fig. 4. The influence of O_2-deficiency on periodic activity, and its influencing by increasing the dissolved O_2-content of water

a — oil layer
b — pouring of 1 litre fresh water under the oil layer
c — removal of $^1/_2$ l water, its oxygenization and replacement under the oil layer.
The data above the actogram indicate the O_2-content of water

On the basis of findings obtained by previous experiments such (a) similar effects of the application of SH-inhibitors as in the case of O_2-deficiency [15] which may be related to the fact that SH-inhibitors are producing practically anaerobe conditions by paralyzing the enzymes that are important in tissue respiration [2] and (b) the elimination of the similar influence of KCl by substances belonging to the tricarbonic acid cycle [7], it was suggested that the essential changes in the mechanism of central nervous system regulating periodic activity are due to influences produced on tissue respiration and on aerobe oxydation processes. Thus the attention was drawn to lactic acid, which — upon the evidence of generally known biochemical data — increases in the tissues under hypoxygenic and anaerobe conditions.

Experiments were performed to examine the changes in lactic acid content at optimal O_2-level, at hypoxygenic respiratory conditions and upon the

application of KCl and SH-inhibitors. Lactic acid determinations were performed in different organs of fresh-water mussels being in various functional conditions, further in animals maintained under O_2-deficient conditions, or treated with KCl or $CdCl_2$.

Lactic acid determinations in the various organs of fresh-water mussel

Lactic acid determinations were carried out in the following organs:

(1) adductor muscles; (2) gill; (3) photosensitive area rich in cilia of the mantle around the siphon; (4) other areas of the mantle. Differentiation of the

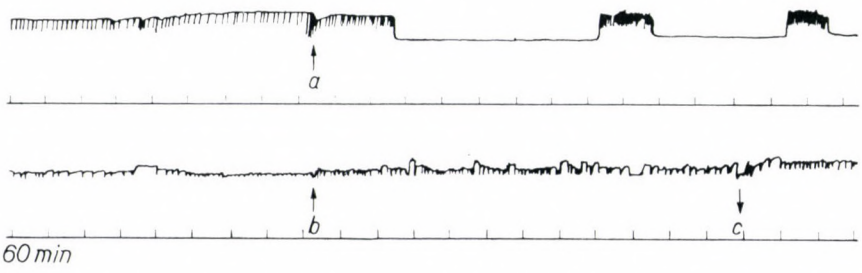

Fig. 5. The effect of denervation on the susceptibility of periodic activity to influences by O_2-deficiency. *a* — oil layer; *b* — oil layer in the same animal after denervation; *c* — change of water

above two areas of the mantle is based on the consideration that the area around the siphon is in direct contact with substances dissolved in water which are entering and leaving the animal. This area has presumably a more important role in chemoreception and thus its functional importance may differ from that of the other areas of the mantle.

Determinations were made in the following groups of animals:

(1) animals displaying lasting activity at high O_2-level, killed in the period of activity;

(2) animals maintained at mediocre O_2-level, and killed just at the transition from lasting closure to the period of activity;

(3) animals maintained at mediocre O_2-level, at the transition from lasting activity to closure;

(4) animals kept for 48 hours under hypoxygenic (< 1 mg O_2/litre) conditions;

(5) animals kept in 1×10^{-3} M KCl solution for 48 hours;

(6) animals kept in 10 mg/litre $CdCl_2$ solution for 48 hours.

The influence of the applied concentrations of KCl and $CdCl_2$ on periodic activity has been examined in the course of previous investigations [7, 15].

The experimental results are summarized in Fig. 6.

As the findings show, lactic acid content (per dry matter) is the highest in the area of the mantle around the siphon. Lower values of lactic acid con-

tent were found in the adductors and in the other areas of the mantle, and it was the lowest in every case in the gill.

No marked differences in lactic acid content exist, even if average values are considered, between different groups of animals. Due to the great dispersion of values it is also impossible to establish a significant difference. Thus, it may be stated that the experimental results obtained were rather unexpected, i.e. there were no differences demonstrable in the lactic acid content in the tissues of animals of different functional conditions. Further, no consid-

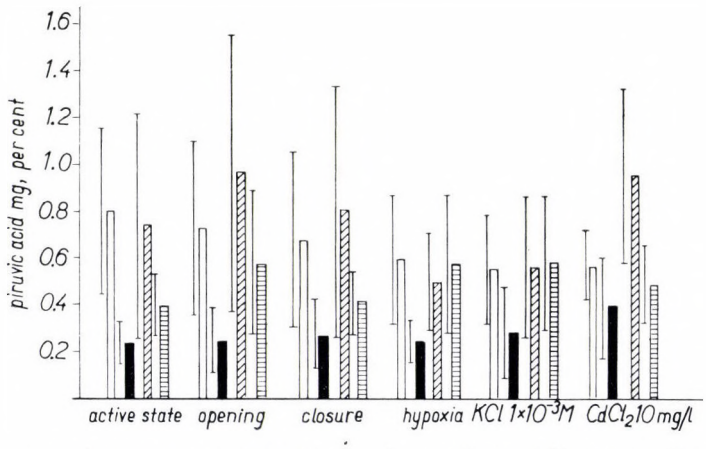

Fig. 6. Lactic acid content in certain tissues of animals in different conditions of activity, or exposed to different effects

□— muscle; ■ — gill; /// area of mantle around the siphon; ☰ lateral part of the mantle. The lines at the columns indicate standard deviation

erable increase was observable in the lactic acid content of the examined tissues either under hypoxygenic conditions or after a pretreatment with SH-inhibitors or KCl.

Discussion

The conclusions to which these experiments lead may be summarized as follows: The O_2-content of water is playing an important or perhaps a leading role in the regulation of this biological rhythm of fresh-water mussel. Both the decrease and increase in O_2-content itself is able to decisively influence the process of periodic activity. Changes in periodic activity in O_2-deficient milieu are connected as a matter of fact with a considerable decrease in the active life functions of the animal. The findings obtained are very interesting also from the biological point of view and their importance is easily understood when it is considered that fresh-water mussel has to conform itself to the extremely varying O_2-levels in the different seasons of the year.

It was also observed that the experimental animals were active in 86% of the day if the water was relatively well supplied with O_2, namely, in the 24 hours before pouring the oil layer on the surface of water. During the 24 hours following this sealing of water surface, activity is reduced to 41% of the day, and the average value of O_2 consumption of the animals is 0.98 mg per hour per 100 g wet weight (body weight without valves). By a decrease of O_2-level, duration of activity becomes restricted to 30% of the day and the amount of O_2 consumed will be 0.14 mg/hour/100 g body weight. If O_2-consumption is referred only to the duration of active periods the amount of consumed O_2 would be 1.11 mg and 0.24 mg/hour/100 g body weight at 10.4 mg/litre and 1.87 mg/litre concentrations respectively. Under the same conditions the number of "changes" within 24 hours was 0.6 and 5.4 respectively (averages of five animals).

The results reported here support the view that changes in periodic activity are very important factors in the adaptation of the animal to O_2-deficiency, and thus they are important also from the biological or ecological points of view.

The influence of O_2-level as a factor determining periodic activity of *Anodonta* manifests itself in a completely different manner from that usual in biological rhythms. Though it is an external factor, still it does not display periodic changes similar to that observed in the case of components determining rhythms regulated by diurnal changes or ebb and tide. The number of alternations between periods of activity and quiescence is increased only by the fall in O_2-content. Thus, the mechanism itself that regulates the "changes" is not activated by the rhythmic fluctuation of environmental conditions, but its scheme of activity is determined by the level of an important external factor.

It is assumable therefore, that the regulation of periodic activity takes place in the following way: the activity of adductors is regulated by corresponding cells in the central nervous system. These elements in the central nervous system display such cyclic metabolic and activity properties developed in the course of phylogeny, which secure the periodic occurrence of relaxation or tonic contraction of the adductors. This fundamental mechanism is influenced by informations arriving from the periphery. The effect of these informations manifests itself in frequency changes of periodic alternations and in the duration of the single periods respectively.

Upon the evidence of denervation experiments the alterations taking place in the periphery and influencing activity arrive into the central nervous system through nervous ways. The present experiments also prove that the stimulus in the periphery is supplied by the level of oxidative metabolic conditions. If it is constant then an approximately regular alternation of periods of activity and quiescence takes place. At optimal oxidative metabolic condi-

tions lasting activity periods are alternating with short rest periods, whereas under hypoxygenic conditions the periodic and surprisingly regular alternation of short activity and more lasting rest periods is observable.

On basis of data obtained so far it is not possible to state as yet in which way and by which direct chemical or other factors oxidative metabolic disturbances are producing their effect. It could be expected that an increase would take place in the lactic acid content of the tissues upon the influence of factors inhibiting hypoxygenic or aerobe respiration. Since these influences may be eliminated by denervation it was thought that the increase in lactic acid content would influence the central nervous elements through the nerves. Nevertheless, not even in the case of animals kept under nearly anoxygenic conditions could accumulation of lactic acid content be detected in the tissues examined. Naturally, this does not imply that there are no important, stimulating increases in lactic acid content taking place in the peripheric elements of the nervous system.

Most noteworthy are in this respect the data obtained by KROMPECHER [8] establishing that in certain tissues of low metabolism the hypoxydative conditions do not manifest themselves in the accumulation of lactic acid but in the production of a special substance, i.e. in mucopolysaccharide formation, both in normal and in pathological conditions. A considerable amount of mucopolysaccharides was detected in the tissues of fresh-water mussel by Zs.-NAGY and LÁSZLÓ [23]. It is assumable on the basis of these findings that the final effect of anaerobe respiration is not manifested in the increase of lactic acid content in the tissues, of fresh-water mussel, but rather — in agreement with the specific metabolic type suggested by KROMPECHER — in the formation and accumulation of mucopolysaccharides. For this reason it is also assumable that the regulation of periodic activity may in a certain way be connected with this interesting phenomenon.

The fact that a similar influence to that of O_2-deficit may be produced by $CdCl_2$ as has been demonstrated by previous experiments [15], may be interpreted as follows: Cd is inhibiting anaerobe respiration by blocking SH-groups and the produced effect is from the point of view of metabolism similar to that produced by O_2-deficiency.

This explanation does not hold true in the case of the similar effect produced by KCl [7], for upon the evidence of previous studies [11] KCl does not essentially influence tissue respiration. In this case assumably the well known, exciting resp. depolarizing effect of KCl is involved.

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SAUERSTOFFVERSORGUNG ALS EIGENTÜMLICHER REGULATOR DES AKTIVITÄTSRHYTMUS DER SÜSSWASSERMUSCHEL *(ANODONTA CYGNEA* L.*)*

Untersuchungen wurden vorgenommen um festzustellen, ob der Sauerstoffmangel die periodische Aktivität der *Anodonta cygnea* beeinflußt. Hierbei zeigte sich, daß die Verminderung des gelösten O_2 Gehaltes des Wassers die Dauer der ununterbrochenen Aktivität verkürzt, und deren Abwechslung mit den Ruheperioden bedeutend steigert. Dieser Effekt hört nach dem Durchschneiden der Nerven der Kiemen und Siphonregion auf. Diese Resultate sowie unsere früheren Angaben, denen zufolge die periodische Aktivität auch mit gewissen auf die Gewebeatmung einwirkenden Substanzen beeinflußt werden kann [7, 15], weisen darauf hin, daß in dieser Regelung die peripherischen Stoffwechselvorgänge, vor allem unter normalen Verhältnissen die Sauerstoffversorgung und die aeroben und anaeroben Vorgänge der mit Wasser in Berührung kommenden Geweben eine Rolle spielen.

Im Verlauf der Untersuchungen wurde kein signifikanter Unterschied zwischen dem Milchsäuregehalt der verschiedenen Organe der in guter Sauerstoffversorgung lebenden und der in zehnmal weniger freies O_2 enthaltenden Wasser lebenden Tieren festgestellt, obwohl der auf 100 g lebendes Körpergewicht berechnete O_2 Verbrauch im 24stündingen Durchschnitt bei den ersteren 1.11 mg/St und bei den letzteren 0.14 mg/St betrug.

In der Regelung dieses biologischen Rhytmus der Anodonten nehmen also nicht periodisch wechselnde äußere Faktoren teil, sondern er ist eine Funktion des freien O_2 Gehaltes des umgebenden Wassers bzw. der in den mit der Umgebung in direkter Verbindung stehenden Geweben sich abspielenden aeroben und anaeroben Atmungsvorgängen.

СНАБЖЕНИЕ КИСЛОРОДОМ — СПЕЦИФИЧЕСКИЙ РЕГУЛЯТОР РИТМА АКТИВНОСТИ ПРУДОВОЙ БЕЗЗУБКИ *(ANODONTA CYGNEA* L.*)*

Изучался вопрос о том, влияет ли недостаток кислорода на периодическую активность беззубки. Установлено, что падение концентрации растворенного в воде кислорода вызывает укорочение непрерывной активности и одновременно значительно увеличивает частоту смены активных периодов спокойными периодами. После перерезки нервов снабжающих сифонную часть мантии и жабрах подобный эффект не наблюдается.

Эти данные а также предыдущие сведения о том, что веществами, влияющими на дыхание тканей можно изменять периодическую активность (Коштоянц и Шаланки 1958, Шаланки 1960 а) указывают на то, что в ее регуляции решающую роль играет окислительный обмен веществ периферических областей, а в природных условиях именно снабжение кислородом этих, непосредственно соприкасающихся с водой, частей животного.

В течении исследований не была найдена достоверной разницы в содержании молочной кислоты тканей, полученных от животных живущих в воде с хорошим и со сниженным содержанием кислорода, несмотря на то, что потребление кислорода было в первом случае 1,11 мг/час/100 г, а во втором случае 0,14 мг/час/100 г свежего веса.

Таким образом, в регуляции биологического ритма прудовой беззубки участвуют не периодически меняющиеся внешние факторы, а она зависит от окислительных процессов периферических органов, которые в физиологических условиях могут ограничиваться содержанием растворенного в воде кислорода.

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Acta Biol. Hung., **15** (3), 311—320 (1965)

STRUCTURAL CHANGES IN THE ADDUCTORS OF THE FRESH-WATER MUSSEL (ANODONTA CYGNEA L.) DEPENDING ON FUNCTIONAL CONDITIONS

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(Received April 20, 1964)

Synopsis

In periods of both relaxation and isometric contraction the adductor muscles of *Anodonta cygnea* resemble the smooth muscle, though they differ from it in several essential structural features. In the state of isotonic contraction they present a picture which was termed previously "spiral muscle", in the state of more advanced contraction, however, a periodicity consisting of dark Q and light I bands is observable, though it might not be regarded as typical striation. It is not possible to classify the adductors either as smooth or striated muscles on basis of their properties, and it would not be correct either to term them as "spiral muscles". With regard to structural changes depending on functional conditions the term "polymorphous muscle" seems to be justified.

Introduction

The phenomenon that the adductors of the fresh-water mussel are capable to perform a relatively rapid phasic activity as well as tonic contraction ("catch-mechanism") lasting for several hours and requiring only little energy has interested physiologists for almost a century. In the course of muscle physiological investigations performed previously [9] and recently [5, 10, 11, 14] the adductors of *Anodonta* have been placed unanimously into the group of smooth muscles. At the same time, however, literary data on the histological structure of the adductors of fresh-water mussel show many discrepancies. According to Margo [8] the adductor is a true striated muscle, and the same statement may be found in the histological manual by Schaffer [12]. Engelmann [4], on the other hand, regarded it as a spiral muscle in which the myofibrils are located along a spiral. Marceau [7] and Brück [3] refused to admit the presence of striated muscles in the adductors and characterized them as smooth muscles. Moreover, recently even the opinion is found in literature [1] that the adductor contains chiefly smooth muscles mixed sporadically with striated elements, this striation having a transversal direction, and a transition of smooth muscle-cells into striated elements being observable.

In order to correctly interpret physiological investigations it proved necessary to find an explanation for the discrepancies in histological data. The present studies are none the less accounted for when taking into consideration that it is hardly probable that these contradictory data originate from wrong observations.

The examinations presented here are based on the assumption that these discrepancies may be elucidated by structural differences occurring in the muscles at different stages of contraction, a fact which has been disregarded by other workers. For this same reason experiments were started with the aim to examine the histological structure of the adductors of *Anodonta* after fixation at different functional states.

Material and method

Examinations were carried out on 10—16 cm specimens of *Anodonta cygnea* kept in an aquarium in streaming Balaton-water in the period from September to January. The functional states of the muscles were the following:

(1) *State of relaxation.* Relaxation of the adductors was achieved by immersing the animal for 5—10 minutes into water at 45—50 C° temperature. This milieu induced relaxation and the valves open wide, but the viability of the animal is not damaged (heat-narcosis). The same effect was obtained by injecting 2% chloralhydrate into the region of the cerebral and visceral ganglions. After relaxation the adductors were fixed in situ.

(2) *State of isometric contraction.* The valves of the living mussel were trepaned at both ends and subsequently put into fixatives. The fixative induced a complete contraction of the adductors, but this contraction was limited by the valves. Then the adductor was fixed in situ in its physiologically possible shortest condition. In the state of isometric contraction the adductors were fixed also after another treatment, namely after placing a 2—3 cm. thick piece of cork between the valves. In this position contraction was likewise isometric, but it was limited even more than under physiological conditions.

(3) *State of isotonic contraction.* The adductors were cut with a scalpel from one of the valves. In this case contraction was not limited by the closing valve, thus the muscle was able to contract isotonically. The adductors contracted to one half on their physiologically minimum length. After transection one portion of the treated adductor was put into fixative immediately while the other portion only after 30 minutes.

The tissues prepared according to the method referred to were fixed in 10% formaldehyde solution and Susa for 4 and 1 days respectively. Subsequently the adductors were cut out and after having been washed in a series of alcohols, methylbenzoate and benzene they were embedded into paraffin and 6 μ longitudinal and cross sections were prepared in series. Staining of sections was performed with the following methods: haemalaun-chromotrop 2R, Van-Gieson and Heidenhain's ironhaematoxylin method.

Results

In native condition two regions may be distinguished in the posterior adductor of *Anodonta cygnea* : a darker "red" part which is located ventrally and constitutes the greater half of the posterior adductor, and a light "white" part which is located dorsally. In the anterior adductor there are similarly two regions distinguishable, a "red" and a "white" region. Essential differences were not observed between these two muscles. It is clearly seen in the histological picture of transversal sections that there are more connective tissues present in the "white" muscles; the perimysium internum is thicker while this muscle is more proportionate than the "red" one. The closeness of myofibrils could not be determined with light microscope, thus it is difficult to decide whether or not the macroscopic differences existing between the two kinds of muscles may be characterized by this factor.

The histochemical picture does not show essential differences in the periods either of relaxation or isometric contraction. The sections resemble as a whole the usual structure of smooth muscles (Fig. 1). The only difference is that the muscle cells are not spindle-shaped but cylindrical to their full lengths, they are not thinner at the ends but adhere with ends little enlarged in the hypostracum (Fig. 2). The cells ending inside the section could not be observed

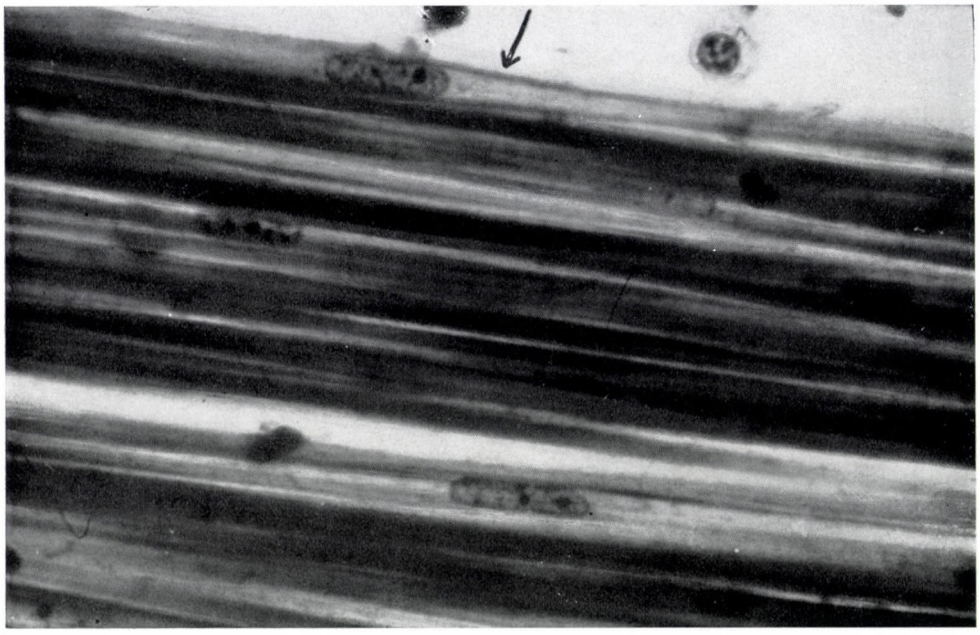

Fig. 1. Longitudinal section of posterior adductor in isometric contraction. Susa-fixation, ironhaematoxylin staining. × 882. Muscle looks like a smooth one ↑ — conical-shaped space without myofibrils at the end of the nucleus

and the visible cell ends are due to the sectioning of cells emerging out of the plane of the sections.

The nuclei are longish, rounded at their ends and located in general in the centre of the cell (Fig. 1). As it was not possible to observe muscle cells at their full lengths, it cannot be stated whether there are perhaps cells with more than one nucleus.

The plasma of the cells seems to be homogeneous in general, in certain regions, however, there are myofibrils observable which are located in tight groups in longitudinal direction on both sides of the nucleus leaving a long cone-shaped space free at both ends. This cone-shaped space stains very little with ironhaematoxyline (Fig. 1). Striation could not be detected in the sections and cells with "spiral" pattern were not found either.

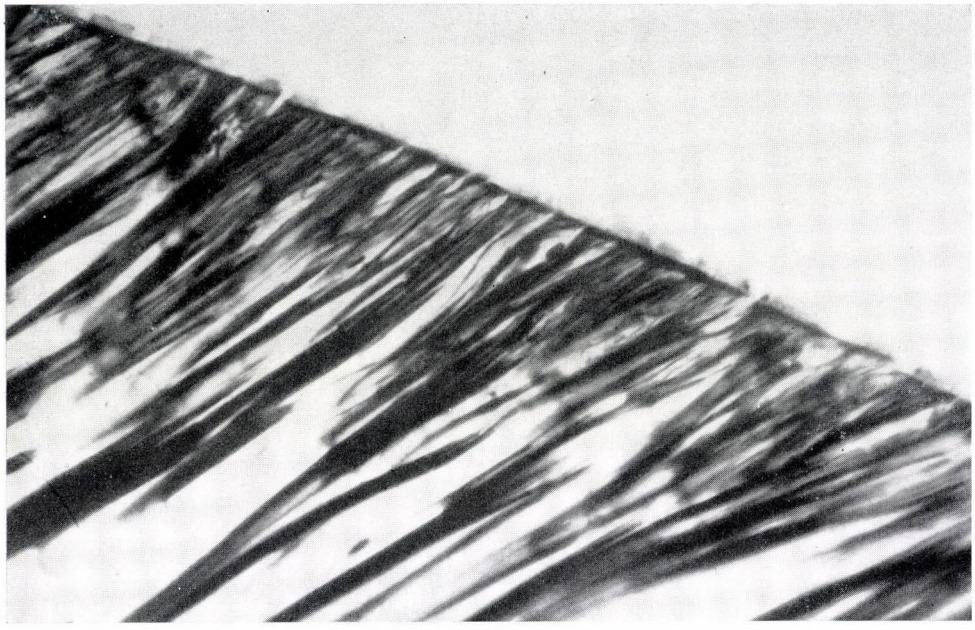

Fig. 2. Adhesion of anterior adductor (in longitudinal sections) in isometric contraction. Fibres adhere with enlarged ends in the hypostracum. Susa-fixation, ironhaematoxylin staining. × 441

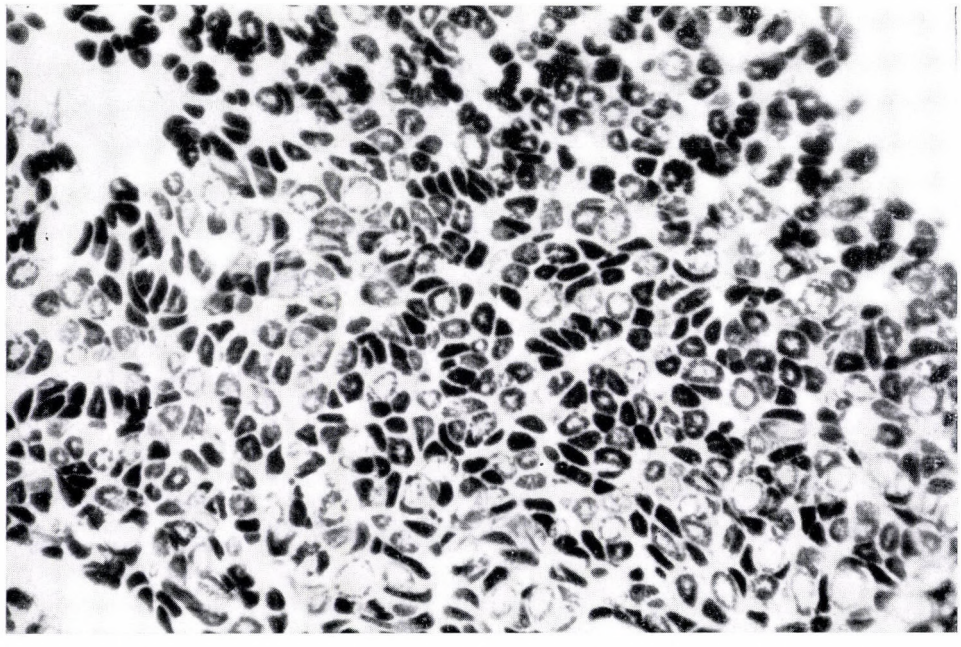

Fig. 3. Anterior adductor in cross-section in the state of isometric contraction. The groups of myofibrils in the lateral region of cells is observable. Susa-fixation, ironhaematoxylin staining. × 441

The adductor presents a very varied picture in the state of isometric contraction. In the greatest part of the posterior adductor the muscle cells appear in the form of rings which are apparently empty inside (Fig. 3). The dimension of this apparently empty middle part varies, the groups of myofibrils are located in the periphery in general, except for some cells which are completely filled with them. Such ring-shaped cross-sections are relatively seldom observable in the posterior adductor.

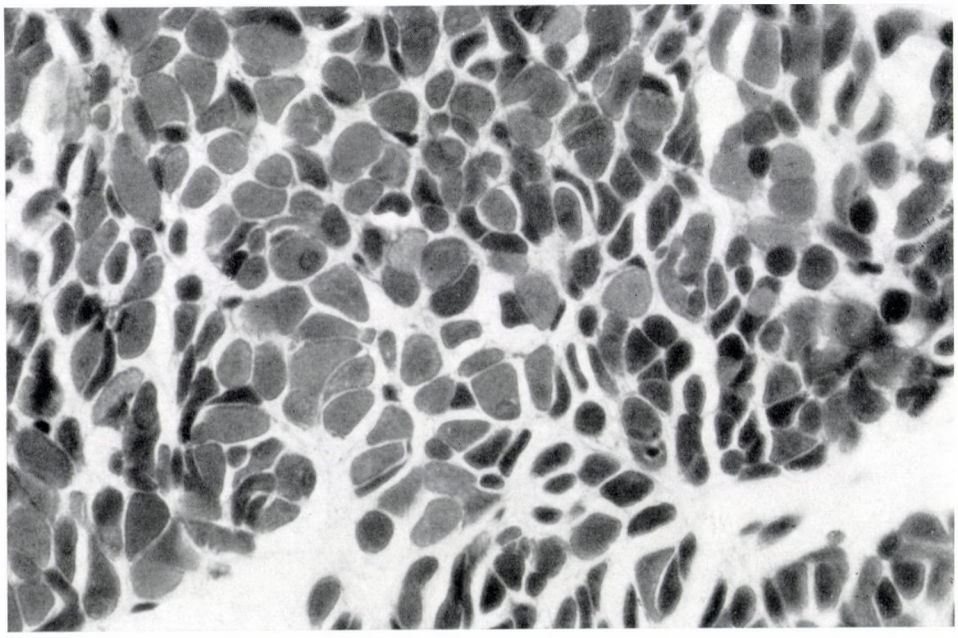

Fig. 4. Anterior adductor in cross-sections in isometric contraction. Homogeneous distribution of myofibrils. Susa-fixation, ironhaematoxylin staining. × 441

The greatest part of the posterior adductor and the smaller, peripheral part of the anterior adductor contains cells which show homogeneous distribution in cross-section (Fig. 4). Their sarcolemma is sharply outlined and their diameter is considerably greater than that of fibres ring-shaped in cross-section.

The histological picture of the longitudinal sections of muscle fixed in the state of isotonic contraction varies depending on whether they were fixed immediately after transection or thirty minutes thereafter. In the muscles prepared immediately the so-called "spiral" structure dominates (Fig. 5), in thin sections, however, it has more a chess-board pattern. Besides these "spiral" fibres cross-striation is also observable sporadically, and is found in nearly every fibre in the longitudinal sections of muscle fixed thirty minutes after transection (Fig. 6). This periodicity consists of about 2 μ wide dark Q

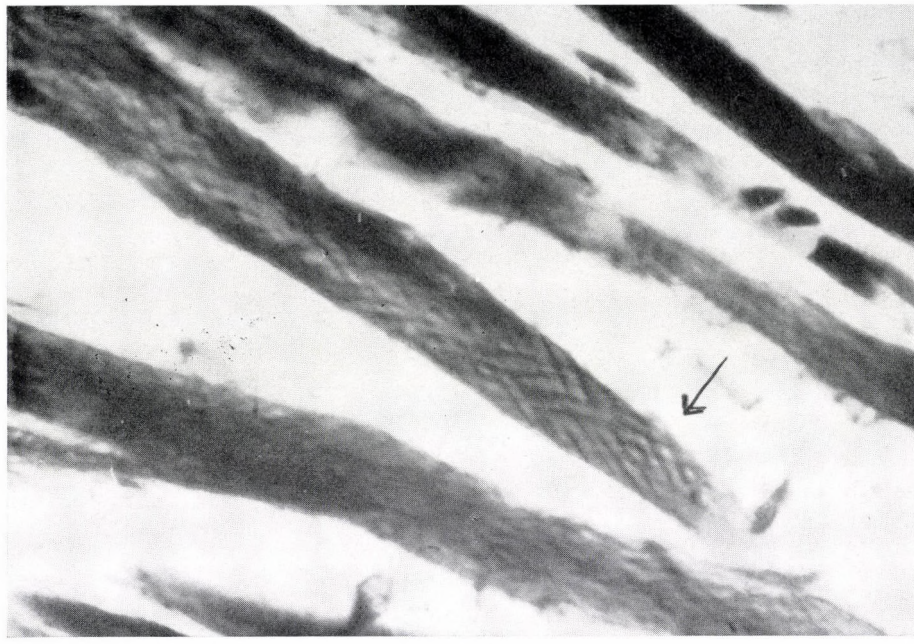

Fig. 5. Longitudinal section of posterior adductor in isometric contraction fixed immediately after transection. ↑-chess-board pattern. Susa-fixation, ironhaematoxylin staining. × 1,260

Fig. 6. Longitudinal section of anterior adductor in isotonic contraction fixed in the 30th minute after transection. Striation is accentuated. Susa-fixation, ironhaematoxylin staining. × 441

and about 3.5 μ wide I bands. The contact line between Q and I bands is not sharp, but dentated, serrated. Bands analogous to other kinds of bands in the striated muscle of mammals are lacking. The position of the bands is in general perpendicular to the longitudinal axis of the fibre, while some others are transversal. Striation is observable in both adductors and no differences were found between the two parts of the adductors.

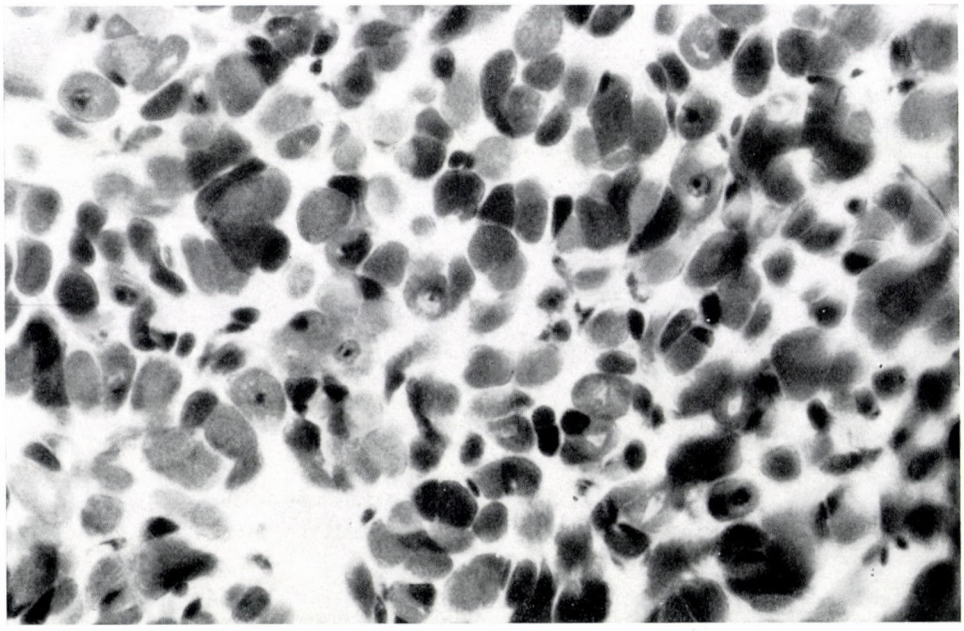

Fig. 7. Cross-section of posterior adductor in isotonic contraction, fixed in the 30th minute after transection. Groups of myofibrils are sharply outlined. Susa-fixation, ironhaematoxylin staining. × 441

In the histological picture of cross-sections the transections of myofibrils are well discernible (Fig. 7). The distribution of the myofibrils inside the cells is apparently homogeneous in the case of both adductors. The cross-sections of cells are not round but often polygonal. In some cases the myofibrils are not discernible in the cross-sections but instead a homogeneous dark spot is visible. The nuclei are located generally in the centre. The diameter of cells is usually greater in normal condition than in the state of isometric contraction.

Discussion

These experiments supply an explanation as to the origin of discrepancies found in literature concerning the structure of the adductors. If the adductors are fixed in the state of either relaxation or isometric contraction,

then in agreement with the findings of MARCEAU [7] and BRÜCK [3] the histological picture obtained will be highly suggestive of smooth muscle. Presumably it was the structure observed in the initial state of isotonic contraction that induced ENGELMANN [4] to term it as spiral muscle. The assumptions, on the other hand, that the adductors might be cross-striated are obviously originating from the cross-striation developed in a more advanced state of isotonic contraction [8]. Moreover very probably the picture of sections fixed in the state of initial isometric contraction was responsible for the idea that there may be both types mixed in the adductor.

As it appears, neither the structural properties nor their changes occurring parallel with various functional stages allow to classify without reservation the adductors of *Anodonta cygnea* as cross-striated or smooth muscles. The adductors cannot be regarded either as muscles with spiral structure on basis of these findings, but rather as specific polymorphous muscles.

It is thought on basis of these experiments that each myofibril of this muscle has a periodicity, but because the cells do not have teleophragma, these periodicities are located at various distances from each other. The about 1,000 Å thickness of myofibrils determined with electron microscope (own unpublished data) is small in comparison to the thickness of some microns of normal histological sections. The periodicities of myofibrils arranged in many subsequent layers can not be detected with light microscope and thus the adductors resemble smooth muscles in the state of isometric contraction. This agrees as a whole with the assumption suggested by LOWY and HANSON [6] in connection with *Mytilus* ABRM.

Isotonic contraction starts immediately after the transection of the adductor. At the outset of this state the myofibrils are arranged in groups in which light and dark elements are located at nearly equal distances above each other. This pattern is visible also under the light microscope, because white and black bands of some thousand Ångströms width are observable even if only few myofibrils get arranged in groups. The periodicities of myofibrils are still located at different levels within the cell, producing a chessboard pattern in the sections. In a more advanced state of isotonic contraction a complete arrangement takes place and striation is observable for the first time. This cross-striation, however, does not bear a strong resemblance to true cross-striation and cannot be regarded as identical with it, because it appears just in the state of contraction and contains only two elements.

The ring-shaped pattern observable in the cross-sections in the state of isometric contraction is likewise unusual in comparison to the histological structure of both smooth and cross-striated muscles. On basis of observations performed on cephalopodes BOZLER [2] assumes that muscle cells having ring-shaped pattern in cross-sections represent non-striated muscle tissues capable of tetanic activity.

In the case of *Anodonta cygnea* it is not possible to decide whether fibres of such pattern in cross-section are capable of true physiologically tonic or tetanic activity, and it is believed that the ring-shaped pattern visible in the cross-sections represent the conical regions without myofibrils observable in the longitudinal sections. It must be emphasized, nevertheless, that the ring-shaped pattern in the cross-sections is not a constant structure, because it does not occur in muscles fixed in the state of isotonic contraction.

In the course of cholinesterase-histochemical studies it was established that the sarcolemma of the adductors contains specific cholinesterase in diffuse granulated distribution [13]. This fact also supplies a basis to distinguish the adductors of the fresh-water mussel from both smooth and striated muscles and further suggests the denomination as "polymorphic muscle" as a means of distinction.

From the above results the following conclusions may be drawn:

(1) The adductors of *Anodonta cygnea* cannot be classified either as typical smooth, or as usual cross-striated muscles. This statement has to be considered in muscle physiological, biochemical or general physiological examinations.

(2) Parallel with functional changes structural changes also occur within the muscle-cells. This is demonstrable with light microscope. This fact may serve as a new viewpoint in researches on the tonic activity of these muscles.

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VOM FUNKTIONELLEN ZUSTAND ABHÄNGIGE STRUKTURÄNDERUNGEN DES SCHLIESSMUSKELS DER MUSCHEL *ANODONTA CYGNEA* L.

Die Schließmuskeln der *Anodonta cygnea* sind dilatiert und in isometrischer Kontraktion der glatten Muskulatur änlich, unterscheiden sich aber auch von ihr in einigen wesentlichen Merkmalen. Zu Beginn der isotonischen Kontraktion zeigen sie das Bild welches früher als spiraler Muskel bezeichnet wurde, bei fortgeschrittener Kontraktion erscheint eine Periodizität von duklen Q und hellen I Streifen. Das letztere Bild entspricht aber nicht einer typischen Querstreifung. Auf Grund der beschriebenen Charakteristika kann der Schließmuskel weder zur glatten noch zur quergestreiften Muskulatur eingeteilt werden, aber auch der Name spiraler Muskel ist nicht gerechtfertigt. Entsprechend der vom funktionellen Zustand abhängigen Strukturänderungen scheint der Ausdruck »polymorpher Muskel« richtig zu sein.

СТРУКТУРНЫЕ ИЗМЕНЕНИЯ, ЗАВИСЯЩИЕ ОТ ФУНКЦИОНАЛЬНОГО СОСТОЯНИЯ, В ЗАПИРАТЕЛЬНОЙ МЫШЦЕ БЕЗЗУБКИ (*ANODONTA CYGNEA* L.)

Запирательные мышцы баззубки в расслабленном состоянии и в изометрической контаракции несколько похожи на гладкую мышцу, но все же отличаются от последней во многих существенных структурных отношениях. В изотонической контракции они вначале напоминают картину раньше т. н. спиральной мышцы, а с углублением контракции на них появляются темная (Q) и светлая (I) полосы, которые однако не соответствуют типичной поперечнополосатости. По своим свойствам заперательная мышца не относится ни к гладким, ни к поперечнополосатым мышцам, но и не отправденно и название её спиральной мышцей. Принимая во внимание структурные изменения в зависимости от функционального состояния, кажется обоснованным употребление названия — полиморфной мышцы.

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Acta Biol. Hung., **15** (3), 321—329 (1965)

MOLECULAR STRUCTURE AND TUMOROUS GROWTH INDUCING EFFECT OF CHLOROSUBSTITUTED PHENOXYACETIC ACIDS IN POTATO TISSUE CULTURE

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(Received June 1, 1964)

Synopsis

The stimulatory influence on growth of phenoxyacetic acid and its disubstituted compounds in potato tissue culture has been studied. When using the applied compounds at concentrations of 10^{-7}, 10^{-4} and 10^{-3} M, the 10^{-4} M concentration was found to have stimulated the growth of tissue cultures. The tissues treated with 2,4-D exhibited the greatest measure of tumorous growth while the stimulatory influence of 3,4-D was somewhat weaker and 2,5-D had a considerably lower stimulatory effect. 2,3-D, 2,6-D and 3,5-D at a concentration of 10^{-4} M failed to present any stimulatory influence on growth. The dichlorosubstituted phenoxyacetic acids at a concentration of 10^{-7} added to 10^{-4} M 2,4-D did not influence the effect of 2,4-D, whereas at a concentration of 10^{-4} M they turned out to be slightly inhibitive, and 3,5-D was even explicitly antagonistic. Growth was found to have been completely inhibited by 10^{-3} M concentrations of various dichlorosubstituted isomers along with 2,4-D at a concentration of 10^{-4} M. MCPA stimulated growth (similarly to 2,4-D), but CMPA had no effect whatever. This indicates that 4-chlorosubstitution plays a major role in the growth inducing effect. An appropriate parallel can be drawn between the stimulatory influence of substituted phenoxyacetic acid isomers on the growth of potato tissue cultures and their auxin effects of different character, such as influence on abscission, herbicide effect, etc.

Introduction

Auxin herbicides are synthetic plant regulators the biological effect of which is from various points of view similar to that of natural hormones [17]. Their influence on organism consists partly of a normal and partly of an abnormal stimulatory effect on growth [5], but no unequivocal hypothesis concerning their primary action has been established so far.

An analysis of the relationship between biological effectiveness and chemical structure reveals that of the biologically active compounds certain materials, the chemical structure of which is entirely different, may exercise an apparently identical influence [27]. At the same time, however, the effectiveness of biologically active compounds can be abolished by changing a radical or an atom [14], or there can be an essential difference in the intensity of regulators which are of almost identical structure [22].

From these statements it may concluded that the growth regulating hormones exercise at first an aspecific physicochemical effect and the idea that this influence manifests itself in the structural change of the cell membrane [13], or in that of the mitochondrium membrane [15] is not of recent origin.

It is well known that the structure of plasmatic membranes influences the transport of exogenous substances. For this reason only materials having hydrophilic-lipophilic properties [29] are capable of permeating in an ideal manner. When examining the influence of phenoxy derivatives on growth and their effect on plant metabolism, it appears that this property is dependent on the quality of the substituents [24] located in the benzene ring, on their number [6] and position [22] and on their place as related to each other [20].

It has been established at various stages of plant metabolism that of the synthetic plant regulators it is the chlorosubstituted derivatives of phenoxyacetic acid, in particular 2,4-D, that exercise an influence similar to, but generally more explicit than, that of physiological auxins [3]. The majority of the experiments were made with whole plants or isolated plant organs [2, 8] with an effect modified also by the conditions of permeating the cuticle.

Earlier we reported that the growth of potato tissues can be specifically stimulated by 2,4-D [10]. Therefore potato tissue cultures appear to be particularly suitable for examining the physiological effects of chlorosubstituted phenoxyacetic acid derivatives.

In the present work the action of 2,4-D auxin herbicide and its analogues was studied on the basis of their influence on growth of potato tissue cultures.

Material and method

Tissue cultures were prepared from tubers of an auxin herbicide-sensitive variety of *Solanum tuberosum* "Gül Baba". Pieces of 6 × 6 × 0.4 mm (25 mg fresh weight) were excised under sterile conditions by applying the previously described method [9]. The tissue pieces were placed on media containing a modified basal nutrient of WHITE [18], 0.05% enzymatically prepared casein hydrolysate („Amparon"), 2% sucrose, 1% washed agar-agar and auxins respectively. The auxins were dissolved at a temperature up to 50° C, adding small amounts of $KHCO_3$ under continuous stirring. The media were adjusted to pH 6.4 with $KHCO_3$.

To compare the effect of 2,4-dichlorophenoxyacetic acid its chlorosubstituted analogues in the induction of tumorous growth of potato tissue cultures, latin square was applied as experimental design. Concentrations of 2,4-D and of analogous substances varied between 0 and 10^{-3} M as presented in Fig. 1.

The auxins tested were: 2,3-dichlorophenoxyacetic acid (2,3-D), 2,5-dichlorophenoxyacetic acid (2,5-D), 2,6-dichlorophenoxyacetic acid (2,6-D), 3,4-dichlorophenoxyacetic acid (3,4-D), 3,5-dichlorophenoxyacetic acid (3,5-D), 2-methyl-4-chlorophenoxyacetic acid (MCPA), 2-chloro-4-methyl-phenoxyacetic acid (CMPA), 4-chlorophenoxyacetic acid (4CPA) and phenoxyacetic acid (PA) respectively.

The chemicals were obtained by the courtesy of J. SÜDI of the Institute of Plant Protection, Budapest. Their identity and purity were examined by chromatographical and biological tests [26].

Each of the experimental variants i. e. subdivisions of the latin square, were represented by 80 to 100 tissue pieces originating from 3 to 6 independent series.

The growth of the tissue pieces was measured by the fresh weight of 14- day old cultures. Growth pattern of the tissue cultures was photographically recorded.

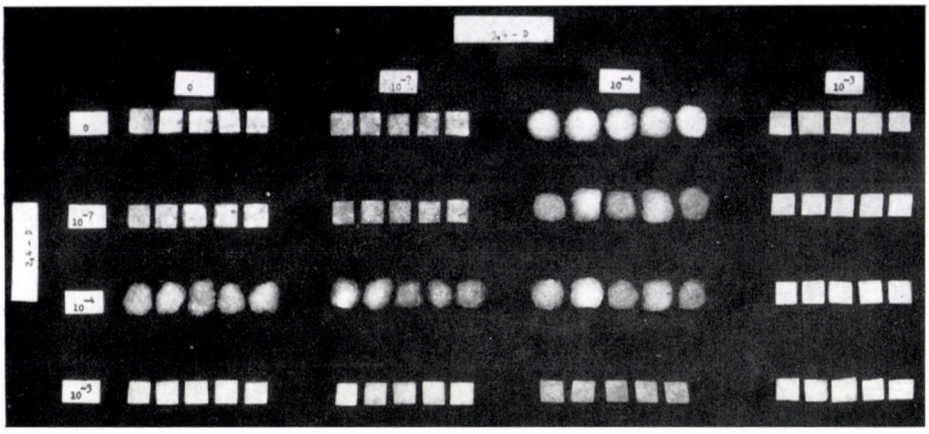


Fig. 1. Experimental layout of the investigation analysing the interaction between 2,4-D and 3,4-D

Results

Comparing the effects of phenoxyacetic acid and its dichlorosubstituted derivatives on each of the compounds used, it was found characteristic that applied at a concentration of 10^{-7} M they failed to stimulate the growth of tissue and concentrations of 10^{-3} M were even found to have considerably inhibited growth. The concentration nearest to optimum was that of 10^{-4} M.

The picture of tissues grown on a nutrient substratum containing an amount of 10^{-4} M of phenoxyacetic acid and its dichlorosubstituted compounds and the changes in their fresh weight are presented in Fig. 2.

It can be concluded from Fig. 2 that neither phenoxyacetic acid nor 2,3-D, 2,6-D and 3,5-D stimulated the growth of tissues. The dark brown colour and weight of the 14-day old tissues are similar to those of the control. Cultures grown on nutrient media containing 2,4-D showed the largest measure of tumorous growth. They are light brown and translucent like glass, four times heavier than the control tissues of similar age. In the case of 3,4-D the stimulatory effect of which was somewhat lower the tissues were flatter and less translucent. Under the influence of 2,5-D which induced the lowest level of growth, the edges of the tissue cultures curled up indicating a type of growth different from the previous ones.

On this basis it may concluded that one of the factors of the effectiveness of the applied compounds on the growth of tissues can be such a molecular charasteristic (electron density, polarity, molecule diameter) which is dependent on the position of the chlorosubstituents of the benzene ring.

In order to obtain some information about the mutual relations between the effects of different dichlorophenoxyacetic acids, we examined the pattern

of the influence of the dichlorosubstituted isomers on the action of 2,4-D which showed the largest and most steady effect.

Different concentrations of dichlorophenoxyacetic acid isomers were combined with 2,4-D at various concentrations. Examination of the possible

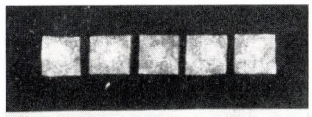
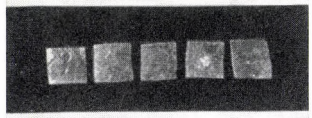
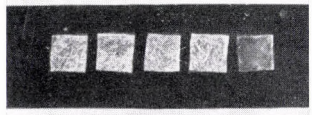
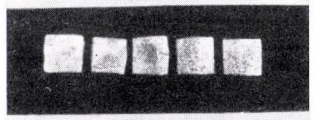
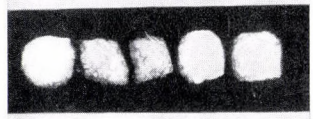
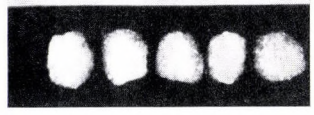
	Material	Mg fresh weight	Weight gain %
	PA	35± 3.0	0
	2,3-D	36± 4.6	0
	2,6-D	38± 3.6	0
	3,5-D	35± 2.8	0
	2,5-D	80±18.5	129
	3,4-D	101±14.6	189
	2,4-D	111±19.6	218

Fig. 2. The effect of phenoxyacetic acid and its dichlorosubstituted compounds (10^{-4} M) on the growth of potato tissues. % weight gain related to the weight (35 ± 4.7 mg) of culture containing no auxin

combinations revealed that an interaction liable to be evaluated could only be obtained when addition was made to 2,4-D at 10^{-4} M concentration.

This interaction is presented in Table 1.

It appears from Table 1 that the dichlorosubstituted phenoxyacetic acids given at a concentration of 10^{-7} M had no effect on the stimulatory influence of 2,4-D. Isomers at a concentration of 10^{-4} M were found to have

Table 1

The interaction between 10^{-4} M 2,4-D and dichlorophenoxyacetic acid isomers of different concentrations (mg fresh weight)

Material	Concentration			
	0 M	10^{-7} M	10^{-4} M	10^{-3} M
2,4-D+ 4-CPA	119±17.1	127±12.7	106±20.9	95±10.8
2,4-D+ 2,3-D	115±13.7	124±13.4	108± 8.8	36± 8.5
2,4-D+ 2,6-D	94± 6.4	90±11.4	83±16.0	43± 5.7
2,4-D+ 3,5-D	124±13.4	131±16.4	80± 4.8	25± 1.2
2,4-D+ 2,5-D	113±17.4	109±20.2	101±24.3	43±13.2
2,4-D+ 3,4-D	111±19.6	119±18.3	96±10.7	37± 5.4
2,4-D+ 2,4-D	106±12.9	—	82± 5.9	—

reduced the stimulatory effect of 2,4-D which was most pronounced when applying 3,5-D.

Dichlorophenoxyacetic acid isomers added at a concentration of 10^{-3} M to 2,4-D of 10^{-4} M caused an inhibition similar to that of 2,4-D which is characterized by toxic concentration. Phenoxyacetic acid, however, reduced the stimulatory influence of 2,4-D even at this concentration to a lesser extent.

Since most intensive growth was obtained with 2,4-D and 3,4-D, the question arises to what extent the stimulatory influence is dependent on the chlorosubstitution of position No. 4, and which way the second chlorosubstituent is involved.

In an attempt to approach the problem the effects on the growth of potato tissues of 2-methyl-4-chloro (MCPA), 2-chloro-4-methyl (CMPA) and 4-chloro(4-CPA) phenoxyacetic acid were compared as presented in Table 2.

From Table 2 it appears that MCPA at the concentrations applied shows an effect similar to that of 2,4-D. In the presence of CMPA, however, the tissue cultures failed to grow altogether. The behaviour of these two substituted phenoxyacetic acids evidently indicates the importance of the 4-chlorosubstitution. 4-chlorophenoxyacetic acid, however, stimulated growth to a lesser extent (60%) than 2,4-D which points to the necessity of filling the second position in order to produce maximum auxin effect. This is not necessarily required to be a chlorine atom.

Table 2

The stimulatory effect of methyl- and chlorosubstituted phenoxyacetic acid isomers on the growth of potato tissues

Material	Concentration			Weight gain* %
	10^{-7} M	10^{-4} M	10^{-3} M	
MCPA	38±4.6	100±15.8	38±5.6	185
CMPA	38±5.7	37± 5.3	42±4.2	0
4-CPA	44±4.2	75±14.2	44±6.2	114

* % weight gain as related to the weight (35±4.7 mg) of culture containing no auxin.

Discussion

The examination of phenoxyacetic acid and its disubstituted compounds in tissue cultures under controlled conditions facilitates the interpretation of their role in growth on the molecular level. Our experiments indicate that the extent to which phenoxyacetic acid and its disubstituted compounds influence the growth of potato tissues is different. In the interpretation of data, the fact that the various chlorosubstitutions change the size of the ring, its electron density, polarity and lipophilia [28] must be taken into consideration. In contrast to dichlorosubstituted analogues, phenoxyacetic acid failed to indicate a stimulatory influence on growth in any of the concentrations applied; this is in accordance with data in literature [25]. It is quite obvious then that chlorosubstitution is essential for effectiveness.

There is no linear relationship between the numerical increase of chlorosubstituents and the intensity of auxin effect [4].

It has been demonstrated [16] that of the trisubstituted phenoxyacetic acids only the 2,4,5, and of the tetrasubstitutions only the 2,3,4,5 are efficient. The other isomers have no effect whatever, or perhaps they are of inhibitive character. It follows then that the effectiveness is determined by the position of the substitution. The largest measurable difference in growth was found between the phenoxyacetic acid and its three disubstituted derivatives, 2,4-D, 2,5-D and 3,4-D. DAY [7] concluded that the 4-chlorophenoxyacetic acid is the most effective of the monochlorosubstituted phenoxyacetic acids. The other 2-chlorosubstitutions increase the mode of action in spite of the fact that the 2-chlorophenoxyacetic acid is of no effect whatever. The 2,4-D thus formed is capable of bringing about such a high measure of stimulation of growth that it cannot be further enhanced either by a change in the position of the substitution or by the numerical increase of the Cl-atoms. A similar picture has been obtained in our potato tissue culture experiment, with 2,4-D having the greatest stimulatory influence on tumorous growth.

3,4-D is characterized by an almost similar effectiveness while 2,5-D, which introduces only a minor change in the steric picture of the molecule, shows a much less stimulatory effect. 2,6-D has no effect whatever on growth. In the first approach this phenomenon seems to be in good agreement with what is known as the two point attachement theory [19] according to which the COOH group of the side chain as well as the free "ortho" position of the ring are indispensable for adhesion to the cytoskeleton. This conception of historical interest has repeatedly been cropping up in recent papers [11] in spite of the fact that it has not been properly supported by experimental evidence.

The experiments conducted by OSBORNE [21] with propionic acid and those of PYBUS [23] with different chlorosubstituted phenoxyacetic acids, benzoic acids and phenylacetic acids refute the theory of two point attachment. VAN OVERBEEK [28] suggests that the substituted 2,6-D hampers the fitting in at an appropriate depth in the lipoid layer on account of the diameter of the molecule, and for this reason the COOH may not reach a level necessary for exercising an influence.

HANSCH and MUIR [12] assume that efficiency requires a free "C" atom opposite the "ortho" position. This statement has been verified by our experiments because in addition to 2,4-D, 3,4-D is also effective. It seems likely that in case of 3,5-D the enlarged diameter of the molecule composed of "Cl" atoms facing each other may cover those parts of the lipoproteid membrane which could be the special area of effectiveness of 2,4-D.

It can be concluded from our experiments conducted with various disubstituted phenoxyacetic acids that the 4-chlorosubstitution has a specific stimulatory influence on growth. As far as the role of the 2-chlorosubstitution is concerned we suppose that it fixes the molecule on the appropriate space of effect.

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MOLEKULARE STRUKTUR UND TUMORÄHNLICHE WACHSTUMFÖRDERNDE WIRKUNG DER PHENOXYESSIGSÄURE UND IHRER DICHLORSUBSTITUIERTEN VERBINDUNGEN IN KARTOFFEL-GEWEBEKULTUREN

Die wachstumfördernde Wirkung der Phenoxyessigsäure und ihrer dichlorsubstituierten Verbindungen in Kartoffel-Gewebekulturen wurde untersucht. — Bei Dosierung der verwendeten Verbindungen in 10^{-7} und 10^{-4} Mol Konzentration wurde festgestellt, daß das Wachstum der Gewebekulturen von der 10^{-4} Mol Konzentration gefördert wird. Das größte tumorähnliche Wachstum wurde bei den mit 2,4-D behandelten Geweben festgestellt und neben dem eine etwas geringere fördernde Wirkung ausübenden 3,4-D zeigt 2,5-D eine geringere Wirkung. Die 10^{-4} Mol Konzentrationen von 2,3-D, 2,6-D und 3,5-D fördern das Wachstum der Gewebe nicht. — Betreffs der den 2,4-D Effekt beeinflussenden Wirkung der dichlorsub-

stituierten Isomeren zeigte sich (Tab. 1), daß die dem 10^{-4} Mol 2,4-D zugegebenen dichlorsubstituierten Phenoxyessigsäuren in einer 10^{-3} Mol Konzentration die wachstumfördernde Wirkung des 2,4-D nicht beeinflussen. Konzentrationen von 10^{-4} Mol vermindern bereits die Wirkung von 2,4-D einigermaßen, und 3,5-D übt sogar eine ausgesprochen antagonistische Wirkung aus. Von den einzelnen dichlorsubstituierten Isomeren wird in 10^{-3} Mol Konzentration die Wirkung des auf das Wachstum optimalen (10^{-7} Mol) 2,4-D gehemmt, die Phenoxyessigsäure weist jedoch auch in dieser Konzentration nur eine geringfügige Hemmung auf. In unseren — auf die wachstumfördernde Wirkung der 2 und 4 chlorsubstituierten Verbindungen der Phenoxyessigsäure bezüglichen Untersuchungen wurde die auf das Gewebswachstum ausgeübte Wirkung von MCPA, CMPA und 4-Cl Phenoxyessigsäure studiert und festgestellt, daß MCPA eine ähnliche wachstumfördernde Wirkung ausübt wie 2,4-D, während CMPA wirkungslos ist. Dies verweist darauf, daß in der Wachstumförderung die 4-Cl Substitution eine bedeutende Rolle spielt.

МОЛЕКУЛЯРНАЯ КОНФИГУРАЦИЯ И СТИМУЛИРУЮЩЕЕ ОПУХОЛЕВИДНЫЙ РОСТ ДЕЙСТВИЕ ХЛОРЗАМЕЩЕННЫХ ФЕНОКСИУКСУСНЫХ КИСЛОТ В КУЛЬТУРЕ ТКАНЕЙ КАРТОФЕЛЯ

Авторы изучали стимулирующее рост действие феноксиуксусной кислоты и ее дихлорзамещенных производных на культуре тканей картофеля. Применяя указанные соединения в концентрации 10^{-7}, 10^{-4} и 10^{-3} М обнаружили, что концентрации 10^{-4} М стимулируют рост культуры тканей. Самый большой опухолевидный рост обнаруживают ткани, обработанные производным 2,4-D; кроме оказывающего несколько меньшее стимулирующее действие 3,4-D, производное 2,5-D оказывает только минимальное действие. Концентрации 10^{-4} производных 2,3-D, 2,6-D и 3,5-D не стимулируют рост тканей. Относительно влияющего на акцию 2,4-D действия отдельных дихлорзамещенных изомеров, авторы нашли (таблица 1), что прибавленные к 10^{-4} М, 2,4-D дихлорзамещенные производные феноксиуксусной кислоты в концентрации 10^{-7} М, не влияют на стимулирующее рост действие 2,4-D. Те-же соединения в концентрации 10^{-4} М уже в небольшой степени снижают акцию 2,4-D, а производное 3,5-D уже оказывает даже выраженно определенно антагонистическое влияние. Отдельные дихлорзамещенные производные в концентрации 10^{-3} М уже полностью тормозят оптимальное (10^{-4} М) для роста действие 2,4-D, однако феноксиуксусная кислота и в этой концентрации оказывает только незначительное торможение. В исследованиях, направленных на выяснение эффективности замещения хлором во 2-ом и 4-ом положении по отношению стимулирования роста, авторы изучали действие МХФК, ХМФК и замещенной хлором в 4-ом положении феноксиуксусной кислоты на рост тканей. Обнаружили, что МХФК оказывает похожее на 2,4-Д действие, а ХМФК является неэффективной. Этот факт указывает на то,что в стимулировании роста большую роль играет замещение хлором в 4-ом положении.

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Acta Biol. Hung., **15** (3), 331—336 (1965)

STUDY OF THE HEXOSAMINE PRODUCTION OF RAT LIVER MINCE UNDER ANAEROBIC AND AEROBIC CONDITIONS

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(Received June 15, 1964)

Synopsis

According to numerous data in literature a correlation seems to exist between the poor oxygen supply of the tissues and their high mucopolysaccharide content. In experiments performed on rat liver mince the author investigated whether any difference could be observed *in vitro* in the anaerobic and aerobic hexosamine production. The liver mince was shaken in Krebs-Ringer bicarbonate buffer solution containing glucose, in the presence of glutamine, at 37° C, in nitrogen atmosphere and in air respectively. The determinations of tissue hexosamine according to Boas' method performed at zero time and after 3 hours showed an increase of 16.6 per cent of hexosamine content under anaerobic conditions whereas only an increase of 4.6 per cent was measured under aerobic conditions.

Introduction

As it has been pointed out by numerous authors, tissues with high muco polysaccharide content have a poor oxygen supply and fermentation meta bolism. Such are the tissue cultures, except for those of mesenchymal origin [8, 21] and the poorly vascularized "bradytrophic" tissues of the adult organism: cartilage, tendon, vitreous body, cornea [6]. Since WARBURG [22, 23] the tumors are known to have a glycolytic metabolism of a generally high mucopolysaccharide level. An exception to this is the Jensen sarcoma in which a high glycolytic activity was found but no mucopolysaccharide production [8].

On the basis of examinations on fibroblast cultures GROSSFELD [8] suggested the possibility of a correlation between deficient respiration, anaerobic metabolism and mucopolysaccharide production. BERENCSI and KROMPECHER [1] demonstrated that severe hypoxia is associated with increased mucopolysaccharide content in tuberculosis of the lung [1] and in C_3H mouse cancer [11]. The same correlation was observed in varicose ulcer and thermal injuries [10] as well as in the course of regenerative cartilage formation [9] and in skin rendered hypoxydotic by mechanical influences [19].

These histological and histochemical results obtained in animal experiments and clinical material prompted us to investigate *in vitro* whether the rate of mucopolysaccharide production may indeed be dependent upon the oxygen supply of the tissue.

The first observations on the hexosamine production *in vitro* were described by LELOIR and CARDINI [12] with *Neurospora crassa*. CASTELLANI and ZAMBOTTI [7] using epiphyseal cartilage homogenates to which glutamine and glucose-6-phosphate were added, found increased amounts of hexosamine in the supernatant of the homogenate. Similar results were obtained with costal cartilage, liver and lung, though to a lesser extent. Heart, kidney, spleen and blood were found to be inactive. BOSTROEM and co-workers [5] described hexosamine production in the cartilage, POGELL [15] POGELL and GRYDER [17] in the rat liver, POGELL and KOENIG [16, 18] measured the changes of hexosamine content in the washed cornea in the presence of glutamine. Hexosamine production was demonstrated by WOLF and WARANDANI [25] in the colon, by BOLLET and SCHUSTER [4], SEKHARA VARMA and BACKHAWAT [20] in the connective tissue and by MC GARRAHAN and MALEY [13] in the liver.

POGELL and GRYDER [17] in their experiments on rat liver investigated the hexosamine production in the presence of glutamine and different hexose phosphates. They employed the term "aminotransferase" for the active enzyme and supposed a consequent production of hexosamine-6-phosphate. In their experiments on cornea POGELL and KOENIG [18] found that by adding radioactive labeled glucose also glucosamine and galactosamine may arise. Similarly, an increase of hexosamine content was described in the presence of ATP and NH_4^+ ion (instead of glutamine) though to a lesser degree [4, 13,15, 17].

Method

For the study of hexosamine production the method of POGELL and KOENIG [18] was used with a slight modification. The experimental material was rat liver. The rats were killed by a hit on the nape and the livers were immediately removed. The spleen and connective tissue were trimmed off and the livers — keeping the dish in iced water — were minced thoroughly with a pair of fine scissors. Fast blending in a Waring blendor and higher temperature should be avoided since the enzyme becomes inactivated, displaying a decrease in activity even at $-20°$ C [17]. The hexose-phosphate transaminase — according to our experiments — was not demonstrable in alcoholic-aether powder.

An amount of 0.6—0.9 g of liver mince was placed in each Warburg flask to which 0.5 ml of 1 per cent glucose and 0.4 ml of 0.1 per cent glutamine (about 2.7 mmol.) were added and the mixture was made up to 3.2 ml by adding Krebs-Ringer bicarbonate buffer solution (pH 7.4). The flask attached to the manometer was placed on the water bath of the Warburg apparatus. For anaerobic experiments the tubes were flushed with N_2 containing 5 per cent CO_2. For aerobic experiments the tubes worked in air. The whole series was shaken simultaneously for 3 hours at 37° C, the shaking rate being 70/min. The measurements were made at zero time (controls) and after 3 hours. The material to be measured — possibly the whole quantities — were washed into the tubes supplied with glass stoppers with as much hydrochloric acid as was necessary to obtain a final concentration of 4N. After a careful fastening down of the glass stoppers and hydrolysation for 4 hours as usual, dilution to 24 ml (about 0.8 N HCl) and filtering according to Boas' method, an aliquot from each tube was transferred on a cation exchange column of Dowex 50. The hexosamine freed in this way from the interfering chromogens was eluated with 5 ml 2 N hydrochloric acid of which a quantity of 2 ml was used for the determination. The method of BOAS was here slightly modified as it was to be feared that if separate blanks and standards would be run with each measurement — as recommended by BOAS — the method would not be sensitive enough to the expectable slight differences due to the errors of measurements of the glucosamine standards. The following procedure was

adopted: only one standard and one blank were run with each series. The ion concentration of the standard and blank, as well as that of each tube of the series were adjusted to the highest 4 N NaOH consumption, i.e. first we added 4 N NaOH by drops in the presence of phenolphthalein to establish which tube had the highest consumption. Then the same number of drops of 4N NaCl was added to the standard and blank, using the same burette. The ion concentration of the other tubes was also adjusted to the highest drop number by addition of drops of 4N NaCl. Then neutralization of the material was performed by adding 0.5 N HCl until the pink colour of the slightly alkaline phenolphthalein disappeared and then distilled water was added to obtain the highest number of drops. The same number of distilled water drops was added to the standard and blank alike. In this way we succeeded in obtaining the same ion concentration in the tubes containing the material to be measured, in the standard and blank. As for the later steps we proceeded according to the method of BOAS: we added acetylacetone dissolved in N Na_2CO_3 and kept the tubes for 45 minutes at 89—92° C. After cooling, the colour was developed by adding a hydrochloric-alcoholic solution of p-dimethyl aminobenzaldehyde and photometry was performed at 530 millimicrons.

The hexosamine production was studied in anaerobic and aerobic medium to which glutamine containing buffer was added. In a few cases we performed also measurements without addition of glutamine. In such cases the complete measurement series (with two parallels for each) was the following: (1) control with glutamine at zero time, (2) anaerobic with glutamine, (3) aerobic with glutamine, (4) anaerobic without glutamine. The whole series was shaken simultaneously at constant temperature.

After the reaction was completed the whole reaction mixture was analysed, no acetone powder being prepared from it, i.e. not only the protein-bound hexosamine was determined. We did not, however, measure the glucosamine-6-phosphate content of the mixture probably produced during the reaction [4, 13, 20] but washed off from the Dowex column.

Results

Our results are shown in Table 1 where the values of 13 measurements are given considering only those with glutamine. These values represent the hexosamine content of fresh liver expressed in mg per cent.

The hexosamine content of the livers examined showing a rather wide range of variety (46.2—85.1 mg per cent) the values at zero time were taken as 100 per cent and the hexosamine production was calculated and expressed in the percentage of this value (Table 2).

As it is seen from the measurement of 13 values, the hexosamine content of the rat liver which was 64.7 mg per cent at zero time, increased to 75.4 mg per cent in 3 hours under anaerobic conditions, corresponding to an increase of 16.6 per cent. At the same time under aerobic conditions the hexosamine content increased to 67.7 mg per cent which is an increase of 4.6 per cent.

The results of measurements without glutamine (7 cases of another series) are illustrated in Table 2.

As it is seen in Tables 1 and 2, the mean hexosamine content measured under anaerobic conditions in the presence of glutamine increased to 81.3 mg per cent in 3 hours (an increase of 14.5%). Under aerobic conditions the hexosamine content increased only to 72.5 mg per cent (an increase of 2.1 per cent).

Without glutamine we measured 73.1 mg per cent under anaerobic conditions (increase of 3.0%) and 68.9 mg per cent under aerobic ones (decrease of 3.0 per cent).

Table 1

Changes of hexosamine content of the rat liver in vitro in the presence of glutamine, at 37° C, after 3 hours

At zero time mg%	Anaerobic			Aerobic		
	mg%	Change		mg%	Change	
		in mg%	in%		in mg%	in%
85.1	99.0	+13.9	+16.4	75.5	—9.6	—11.2
66.7	70.0	+ 3.3	+ 5.0	74.7	+8.0	+12.0
53.3	78.1	+24.8	+46.5	58.6	+5.3	+10.0
62.7	68.2	+ 5.5	+ 8.8	64.5	+1.8	+ 2.8
66.8	69.5	+ 2.7	+ 4.0	66.2	—0.6	— 0.9
81.7	92.5	+10.8	+13.3	86.0	+4.3	+ 5.3
81.0	92.0	+11.0	+13.6	82.4	+1.4	+ 1.7
71.0	81.3	+10.3	+14.5	72.6	+1.6	+ 2.2
59.2	67.6	+ 8.4	+14.2	65.2	+6.0	+10.0
54.3	59.8	+ 5.5	+10.0	58.6	+4.3	+ 7.9
57.2	63.7	+ 6.5	+11.4	61.9	+4.7	+ 8.2
46.2	64.3	+18.1	+39.3	54.8	+8.6	+18.6
56.0	74.0	+18.0	+32.2	59.5	+3.5	+ 6.2
Average: 64.7	75.4	+10.7	+16.6	67.7	+3.0	+ 4.6

Table 2

Changes of hexosamine content of rat liver, in vitro with and without glutamine, at 37° C, after 3 hours (in mg%)

At zero time	With glutamine		Without glutamine	
	3 hours		3 hours	
	Anaerobic	Aerobic	Anaerobic	Aerobic
85.1	99.0	75.5	80.0	60.7
66.7	70.0	74.7	71.6	59.8
53.3	78.1	58.6	56.6	65.1
62.7	68.2	64.5	59.3	58.5
66.8	69.5	66.2	67.2	68.6
81.7	92.5	86.0	90.0	86.5
81.0	92.0	82.4	87.0	83.1
Average: 71.0	81.3	72.5	73.1	68.9

These experiments demonstrate that the rat liver mince is able to produce hexosamine, presumably starting from glucose [18] by means of glutamine as NH_2 donor and producing more hexosamine under anaerobic conditions than under aerobic ones. Without glutamine the production is lower but here too, the amount of hexosamine produced under aerobic conditions is more reduced than under anaerobic ones.

Our results obtained under aerobic circumstances without glutamine were not uniform, which is in agreement with the findings of McGarrahan and Maley [13]. Production and consumption (phosphorolysis?) could be noted in about equal proportion. According to data in the literature the degree of hexosamine production in the rat liver *in vitro* is much lower than in the livers of other animals or in other organs of the rat [17].

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UNTERSUCHUNGEN ÜBER DIE HEXOSAMINPRODUKTION DES ZERKLEINERTEN LEBERGEWEBES UNTER ANAEROBEN UND AEROBEN VERHÄLTNISSEN

Im Einklang mit zahlreichen Daten der Literatur scheint eine Korrelation zwischen der herabgesetzten Sauerstoffversorgung der Gewebe und ihrem hohen Mucopolysaccharidgehalt zu bestehen. In Versuchen des Verfassers wurde es untersucht, ob in der Hexosaminproduktion des zerkleinerten Lebergewebes die Hexosaminbildung unter aeroben und anaeroben Verhältnissen verschieden sei. Das zerkleinerte Lebergewebe wurde in der Glucose enthaltenden Krebs-Ringer-Bicarbonat Pufferlösung mit Hinzufügung von Glutamin auf 37° C, in Stickstoff-Atmosphäre, sowie in Luft-Atmosphäre geschüttelt. Die Bestimmung des Hexosamingehaltes der Gewebe nach der Methode von Boas wurde im Null-Zeitpunkt und nach 3 Stunden unternommen. Unter aeroben Verhältnissen fand Verfasser bloß eine 4,6%ige Steigerung, wogegen unter anaeroben Verhältnissen der Hexosamingehalt um 16,6% anwuchs.

ИССЛЕДОВАНИЕ ВЫРАБОТКИ ГЕКСОЗАМИНА ИЗМЕЛЬЧЕННОЙ ПЕЧЕНЬЮ КРЫС В АНАЭРОБНЫХ И АЭРОБНЫХ УСЛОВИЯХ

Многочисленные литературные данные указывают, что может иметь место взаимозависимость между недостаточным снабжением кислородом и высоким содержанием мукополисахаридов тканей. В наших опытах исследовали, имеется ли разница **in vitro** в выработке гексозамина измельченной печенью крыс в анаэробных и аэробных условиях. Измелченную печень в присутствии глютамина при 37 градусов инкубировали при постоянном встряхивании в буфере бикарбоната Кребса—Рингера в атмосфере азота и воздуха. Сразу и пустя 3 часа проведенные определения содержания гексозамина в тканях по Боас показывают, что в анаэробных условиях содержание гексозамина увеличивается на 16,6%, а в аэробных условиях лишь на 4,6%.

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Acta Biol. Hung. **15** (3), 337—341 (1965)

THE EFFECT OF CONTINUOUS LIGHT OR DARKNESS ON THE SUBCOMMISSURAL ORGAN AND GLOMERULAR ZONE OF ADRENALS OF THE WHITE RAT

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Synopsis

On the effect of continuous light for one month — in conformity with other authors — a considerable decrease of the relative weight and of the lipid contents of the organ was established in the epiphysis of rats. Continuous darkness, on the other hand, had not influenced the relative weight and contents of lipids in the epiphysis. The subcommissural organ (SCO) showed in each of three groups an identical picture, thus the SCO of rats lost its reactivity to light still existing in *Amphibia*. On the other hand, atrophy in the epiphysis provoked by light effect had not influenced the glomerular zone of adrenals and therefore it is unlikely that a close relationship would exist — considering the function in connection with light — between the epiphysis and peripheral zone of adrenals.

It is well known from experiments of OKSCHE that epiphysis and SCO of *Amphibia* give a rapid and well defined reaction to light effects [7, 8].

According to other authors the epiphysis of mammals during phylogeny preserved its reactivity to light [3, 4, 11, 12]. However, we cannot discover any data in literature concerning the action of light on the SCO of mammals. On the other hand the epiphysis in FARRELL's opinion produces adrenoglomerulotropin, a substance influencing the glomerular zone of adrenal cortex [1].

However, according to the experiments of FÖLDVÁRI, PALKOVITS and co-workers the aldosteronotropic substance can be demonstrated first of all in the SCO [5, 6, 9, 10].

It was the intention of the present study essentially to answer two quenstions: has the continuous light or darkness an effect in rat not only on the epiphysis but also on the SCO and which way do possible changes of the epiphysis influence the aldosterone-producing zone of the adrenal cortex?

Material and method

In November 1963, 12 white male rats each weighing 180—220 g were placed in a dark and a continuously lighted room for a month. The number of control animals was similarly 12 and these were lighted for 6—18 hours. The light was supplied in both rooms by two F-tubes of 40 W and two opal bulbs of 60 W. The animals received during the experiment a standard diet (grits mixture) and water "ad libitum".

The epiphysis, one of the adrenals and the pineal glands of the ether-killed animals were fixed in 6% formalin. The adrenals of the other side and the cerebral portions which contained the SCO were fixed in Bouin's solution. The weight of the formalin-fixed epiphysis was established by torsion balance. The organ was cut with freezing microtome and the lipid content determined with Sudan red 7 B. The sections which were prepared from the adrenals fixed in Bouin's solution, were stained with haematoxylin and eosin. The SCO was

cut in the sagittal plane and stained with Gomori's paraldehyde fuchsin method and haematoxylin-eosin. The volume of nuclei in the glomerular zone of the adrenals and in the central part of the SCO was measured by the nomogram of FISCHER and INKE [2] according to the formula $\frac{\pi}{6} LB^2$, with a 3000× projectional magnification. The volume of 100 nuclei was determined per animal and organ.

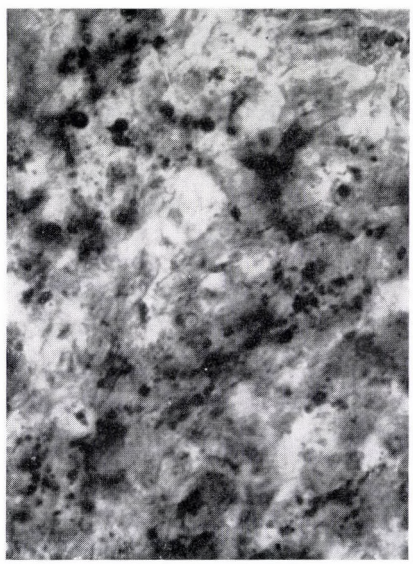

Fig. 1. Mus rattus. Epiphysis, control. Frozen section. Sudan red 7 B stain, × 200

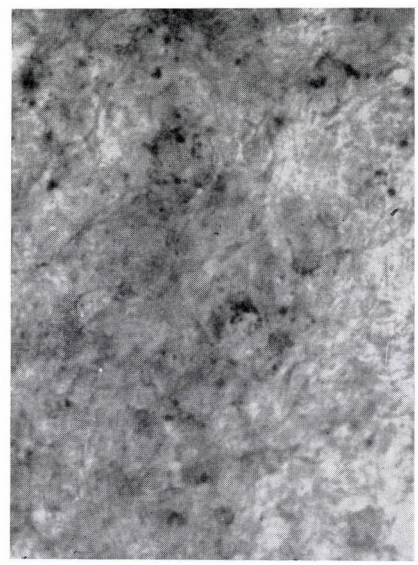

Fig. 2. Mus rattus. Epiphysis. The effect of continuous light on the lipid content of epiphysis. Frozen section, Sudan red 7 B stain. × 200

Results

On the action of continuous light the relative weight of epiphysis decreased to a considerable extent, by 42.5 per cent on the average, in conformity with data of literature. On the other hand the relative weight of the animals kept in continuous darkness showed no difference as compared with the controls (Fig. 1).

On the effect of light the lipid content of the epiphysis considerably decreased (Fig. 2) but did not change on the action of continuous darkness.

Quantity and localization of aldehyde-fuchsin positive neurosecretes in the SCO showed in all three groups an identical picture. The volume-values of nuclei presented no changes as compared with the controls (Fig. 3). Thus the continuous light or darkness was ineffective for the SCO.

Neither light effect nor light deficiency changed sudanophily in the adrenals, similarly there was no change in the course of the curves illustrating the volumes of the nuclei (Fig. 4). Therefore the continuous light or darkness had no appreciable influence on the activity of the glomerular zone.

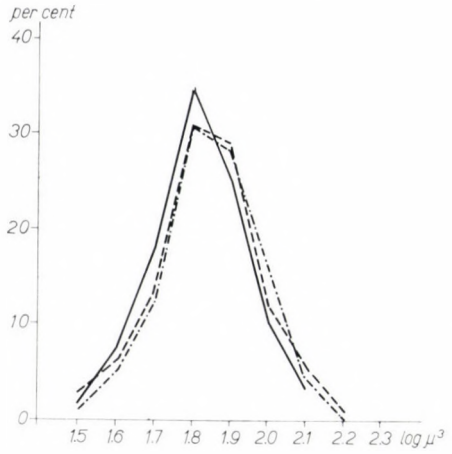

Fig. 3. The effect of continuous light or darkness on the volume of nuclei of the SCO Control ———, continuous light —·—·, continuous darkness --------

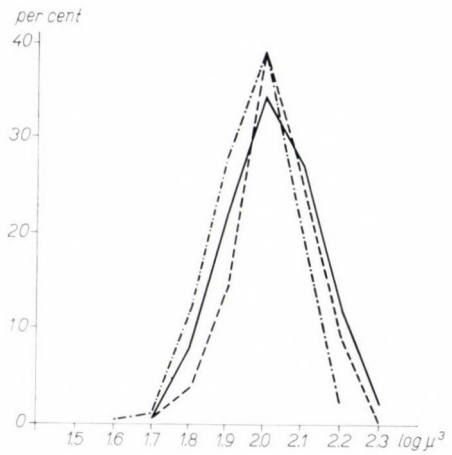

Fig. 4. The effect of continuous light or darkness on the volume of nuclei of the glomerular zone

Discussion

Both epiphysis and SCO underwent considerable morphological changes during phylogenesis. While the epiphysis of *Amphibia* and *Reptiles* contains elements suggestive of light receptors, in birds and mammals it obtains a glandular-parenchymal structure. The SCO of the *Amphibia* is comparatively larger, its cells are abundant in secrete granules in basal position whereas in mammals it is relatively smaller and contains only a few secrete granules chiefly in apical position. Thus the SCO becomes during the phylogenesis more and more smaller and contains ever less Gomori-positive substances [7, 13]. According to the investigations of Oksche the ribonucleic acid content of the epiphysis of frogs decreases on the influence of light to which it was exposed for some days and concurrently the stored ependymal secretes in the basal parts of SCO considerably decrease [7, 8]. On the effect of darkness for a few days an opposite process takes place in both organs. Thus in this case there is a close functional relationship between the two organs.

No similar functional relationship can be observed—according to our examinations — in rats because the atrophy of the epiphysis which was brought about by continuous light was ineffective for the SCO.

It was impossible to conclude on the function of the epiphysis and SCO of *Amphibia* from the light-induced changes. OKSCHE could demonstrate no unequivocal connections between these two organs from the point of view of dermal pigmentation, water-household and osmotic balance of the organism [8].

It is similarly difficult to evaluate continuous light effect-induced atrophy in mammals which was established by other authors, from the functional point of view because the function of the pineal gland is not sufficiently known so far. Should the epiphysis really produce a trophormone which was able to influence the activity of the glomerular zone, then it might be supposed that the atrophy of the pineal gland induced by continuous light could have an effect on the function of the peripheral zone of the adrenals.

However, we have found no morphological signs which vould point to the functional connections between these two organs. On the basis of the present investigations it was impossible to conclude on any relationship between the SCO and the glomerular zone demonstrated by some Hungarian authors [5, 6, 9, 10], because the SCO of rats showed no reaction to the effect of continuous light or darkness.

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ÜBER DIE WIRKUNG DAUERNDER BELICHTUNG UND DUNKELS AUF DIE EPIPHYSE, DAS ORGAN SUBCOMMISSURALE UND DIE ZONA GLOMERULOSA DER NEBENNIERE BEI LABORATORIUMSRATTEN

Es konnte nach einer einmonatigen ständigen Belichtung — entsprechend den Befunden anderer Autoren — ein Rückgang des Lipoidgehaltes und eine bedeutende Abnahme des relativen Gewichtes der Epiphyse bei Ratten festgestellt werden. Werden die Tiere ständig im Dunkeln gehalten so ändern sich der Lipoidgehalt und das relative Gewicht der Epiphyse nicht. Der Befund des subcommissuralen Organs war bei allen drei Gruppen gleich, also hat dieses Organ die bei den Amphibien noch vorhandene Reaktionsbereitschaft gegen Licht verloren. Die durch Lichteinwirkung ausgelöste Atrophie der Epiphyse hatte keinen Einfluß auf die Zona glomerulosa der Nebenniere. Wahrscheinlich besteht — in bezug auf die Lichtreaktionen — kein funktioneller Zusammenhang zwischen Epiphyse und Zona glomerulosa.

ВЛИЯНИЕ ДЛИТЕЛЬНОГО ОСВЕЩЕНИЯ И ТЕМНОТЫ НА ЭПИФИЗ, СУБКОММИССУРАЛЬНЫЙ ОРГАН И НА ГЛОМЕРУЛЯРНУЮ ЗОНУ НАДПОЧЕЧНИКОВ У БЕЛЫХ КРЫС

В эпифизе крыс под влиянием месячного постоянного освещения, согласно другим авторам, наблюдалось уменьшение содержания липоидов и значительное снижение его относительного веса. В то время длительное содержание животных в темноте не оказывало такого влияния. Субкоммиссуральный орган во всех исследуемых группах не изменялся, таким образом он уже потерял способность реагировать на свет, что хорошо выраженно у земноводных. С другой стороны, атрофия гипофиза, вызванная длительным освещением, не оказывает влияния на гломерулярную зону надпочечников, поэтому между эпифизом и гломерулярной зоной надпочечников, вероятно, не имеется функциональной связи.

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Acta Biol. Hung., **15** (3), 343—350 (1965

ALTERATION OF NUCLEIC ACID CONTENT OF DROSOPHILA MELANOGASTER IMAGOES DURING RIPENING AND AGEING

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(Received September 1, 1964)

Synopsis

DNA and RNA contents of the whole body during imaginal stage of *Drosophila* were examined, according to sex. The period of maturation-feeding and egg-ripening is characterized by increasing wet and dry weight, DNA and RNA contents of females. Synthesis of nucleic acid — particularly of DNA — exceeds the synthesis of the other body components, and the RNA/DNA ratio diminishes. DNA content of the males rose, too, but as a contrast the quantity of RNA — and consequently the RNA/DNA ratio — decreased. By beginning of egg-laying (on the 6—12th day of imaginal development) wet and dry weight, and nucleic acid content of females decreases in the same way and subsequently arises to a constant value. In males, the slight decrease of wet and dry weight and RNA content continues, while that of the DNA remains on a constant level. RNA/DNA ratio of *Drosophila* imagoes in the last, ageing period of life did not vary, merely a slight diminishing of the ♀ RNA was found. On the ground of investigations it became clear, that while the first, *ripening period* of imaginal life is characterized by considerable quantitative transformation of the information system, in the course of *ageing* it can not be demonstrated by the method applied. We suppose the latter may be explained in two ways: (a) in the course of ageing the quantitative relationships of nucleic acids are of subordinate importance contrary to *qualitative*, structural modifications, or (b) the information system is submitted to quantitative transformations, too, but in the parenchyma and interstitium, respectively in *different directions*, and so alterations compensate each other.

Introduction

Investigations concerning alterations of the cell information system depending on time, or more exactly on age of organisms are performed at present in two directions.

Qualitative modifications (structural alterations of coding nucleotides, establishment of intramolecular H-bonds, somatic or point mutations, etc.) are known recently as described by DANIELLI [7], HARMAN [13], BJORKSTEN [4], LINDH [18], only approximately. Nevertheless it was demonstrated, e.g. concerning thermal-denaturation [12], that DNA of young animals differs from that of the old ones. This difference can serve as basis for disturbances, developing in synthesis of messenger and cytoplasmic RNA respectively and transformation of codes [21].

Data concerning *quantitative* relationships are highly contradictory. The amount of ribo-, desoxyribonucleic acid and desoxyribonuclease in some organs respectively cell types diminishes, while in others it accumulates or

remains unaltered with increasing age [16, 22, 24, 3, 2]. No authentic data are known concerning modifications of nucleic acid content in the whole organism in animals, subject to ageing.

Material and method

In experiments *vg* strain of the fruit fly, *Drosophila melanogaster*, bred at 25°C, 75 per cent rel. humidity, in darkness, in thermostate was applied. Population-density (10—15 per 100 ccm air volume) was identical; for nutrition the Mainx-medium was used. At the hatching from pupae (0 day) furthermore on the 5, 10, 15, 20 and 25th day of imaginal development the insects were killed with ether and nucleic acid content was registered according to sexes. Quantitative determination of RNA and DNA was performed by the Ogur-Rosen method, from the UV absorption, by means of Uviphot photometer. For each measurement we used 2 × 40 ♂ and ♀ *Drosophila*, resp.all the given values are the averages of determinations of 4 successive series. Besides nucleic acids wet weight and dry matter content (105°C, 24 hours) of the animals were determined, too, according to age groups.

Results

The amount of nucleic acids of the two sexes altered in different ways during imaginal life of *Drosophila*.

RNA content of the females at the hatching from the pupae increased from 0.033 mg by the 5—7th day to 0.050—0.053 mg, per individuum. Subsequently RNA content decreased till the end of imaginal life (Fig. 1). Consequently during the period of maturation-feeding and by beginning of egg-laying the amount of RNA follows the alterations of the wet and dry weight (Fig. 2). Later on, however, the decrease of RNA continues, while total weight of the body remains unaltered. In male specimens the case is different. During the first ten days of imaginal life RNA content diminishes, but later, similarly to the wet weight and dry matter content, it remains on a constant level.

The course of the female DNA curve (Fig. 3) coincides with that of the RNA for the same sex, although the increase in DNA, connected with maturation-feeding is of a slightly higher degree. DNA content of the males, contrary to the RNA does not exhibit a decrease in the first part of imaginal life, but it even rises in the first 5—7 days. However, in the phase of ageing it is unaltered, too.

Comparing the nucleic acid values, greater amount of both RNA and DNA are present in the ♀, than in ♂ specimens. The difference in ribonucleic acid is greater; RNA content of the ♀ is about threefold that of the males.

Percentual values referred to wet weight worked out as seen in Figs 4 and 5. It became evident that increase of the wet weight of ♀ *Drosophilae*, respectively at first diminishing in the males is caused first of all by the RNA synthesis of similar direction (Fig. 4). The same applies to the DNA alterations exhibited by females (Fig. 5). On the other hand, percentual amount of DNA increases not only until the 5—7th, but to the 10—15th day, which indicates

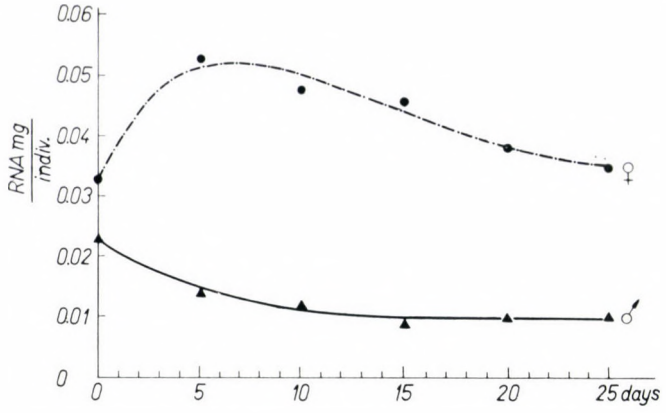

Fig. 1. Alteration of RNA content of *Drosophila melanogaster* during imaginal stage

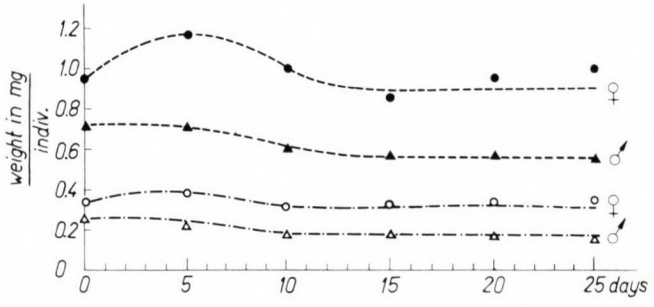

Fig. 2. Wet weight (● ▲) and dry matter content (○ △) of the body of *Drosophila melanogaster* imagoes, dependent on age

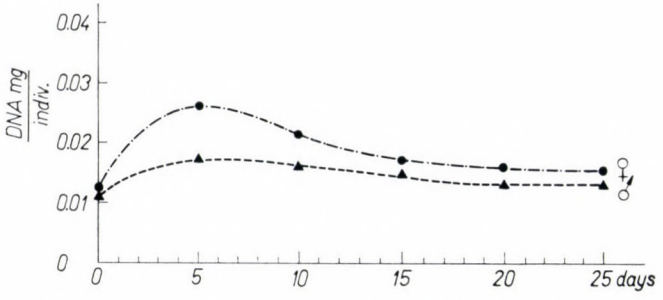

Fig. 3. Modification of DNA content in *Drosophila* during imaginal life cycle

that reduction of DNA in the middle life period of males is of minor extent, than that of other components of the body; however, beginning from the 15th day of imaginal life, the opposite case is valid.

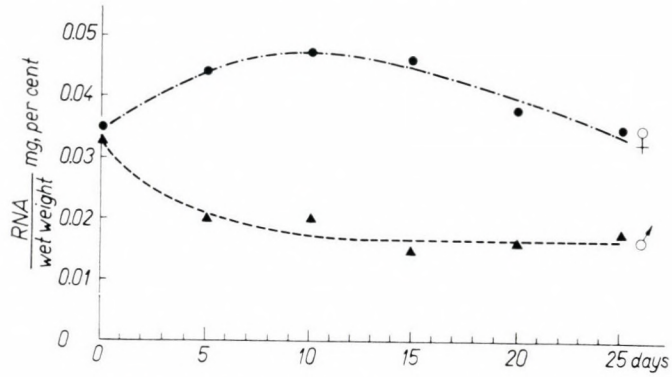

Fig. 4. Alterations of relative RNA content

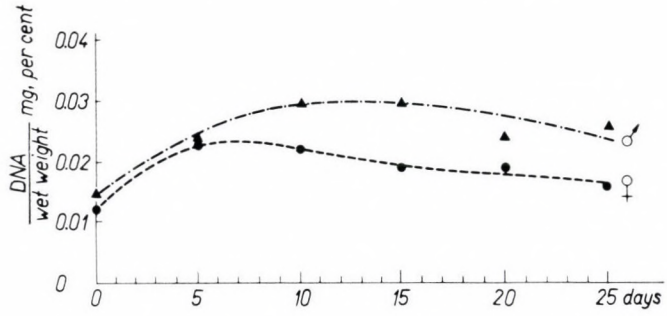

Fig. 5. Alterations of relative DNA content

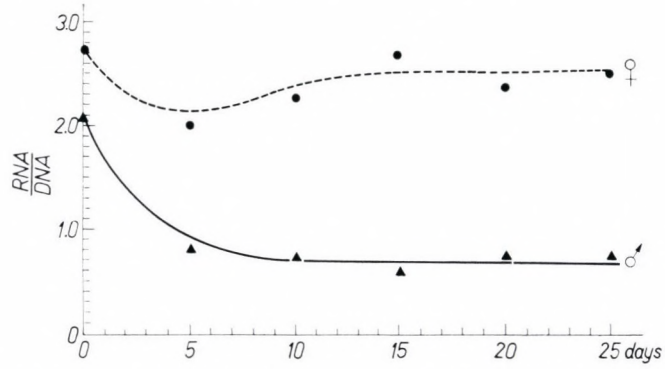

Fig. 6. Modification of RNA to DNA ratio by imagoes of *Drosophila melanogaster*

RNA/DNA ratio, according to the formers develops as follows: in ♂ and ♀ *Drosophilae* the period of maturation-feeding and egg-ripening is characterized by decrease of the ratio (Fig. 6). Diminishing lasts in males until the 7—9th day, but in females, caused by the beginning of egg-laying, it com-

mences to rise on the 5—6th day. In the last, involutional period of *Drosophila* RNA/DNA ratio shows constancy.

Discussion

As proved by our investigations, the information system of the organism is submitted to considerable quantitative transformation in the first days of imaginal life. Although it is age-dependent, it does not represent an ageing-alteration. The imaginal stage of the newly hatched *Diptera* namely cannot be regarded as mature life period, because lasting some days, the imagoes are subjected to *growing* and *ripening* processes. However it is proved by several data, that direction of nucleic acid metabolism during ageing deviates as experienced in the course of maturation. DNA content of the nucleus, e.g. in liver of young rats, rises in the course of growth [15], but exhibits a constant level in ageing of adult animals [10]. According to BÜRGER [5] DNA/dry matter content of the brain diminishes in childhood, remains constant in adults, and begins to rise in senescence. KURNICK and KERNEN [16] measured acid DN-ase activity in thymus and spleen of ♂ mice: they registrated during maturation 3 to 6 fold increase and constant level in ageing.

Data published on the total nucleic acid content of the whole body are also concerned with embryonic development, growth, but never ageing. DAWBARN [8] observed decrease in RNA content of the body in mice. PATTERSON [19] demonstrated that RNA/DNA ratio by the beginning of pupation of *Tenebrio* is high, then low in the middle period, and rises again by the completion of histogenesis; the periods coincide with intensity of protein synthesis.

Concerning *ageing*, that is the latest life period of *Drosophila*, in our own measurements it was striking that neither in absolute amount of nucleic acids, nor in RNA/DNA ratio variations of high degree, similar to maturation, could be observed. This doesn't mean, of course, the unchanged character of the amount of information storing and transforming macromolecules during ageing. Presumably some organs and cell-types modify in different directions and the resultant of these modifications is represented in the constant RNA/DNA ratio of the whole organism. From this standpoint is rational to compare our measured values with detailed data, obtained in other animals.

Undoubtedly, in the organs and cells examined the amount of nucleic acids, first of all of RNA *decreases* in the course of ageing. This was demonstrated e.g. by BÜRGER [5] in human brain, WULFF, QUASTLER and SHERMAN [23] in cerebellar Purkinje-cells of mice, and by WULFF, PIEKIELNIAK and WAYNER [22] in pyramidal cells of rat forebrain cortex. In other cases, inversely, measurements proved the increase of RNA (rat liver: DETWILER and DRAPER [9]; liver cells and dorsal root cells of mice: WULFF, QUASTLER and SHERMAN [23]), or constant level independent of age. At present we cannot supply a suitable explanation, why RNA concentration modifies according to individual

organs and cells with increasing age in opposite directions. Generalization is hindered by racial deviations. BARROWS, ROEDER and FALZONE [3] observed in the liver of ♀ wild rats an increase, while in laboratory albino rats a constant level of RNA content. Origin of these gerontological differences becomes more evident, if the deviations in nucleoproteids of the single species, independent of age [20] will be known in detail.

Causal understanding of the process became easier by the experiments performed with incorporation of H_3-labelled citidin in mice of different ages. It was proved, that the rate of incorporation grows faster, or decreases slower in ageing, than RNA content itself. The tracer uptake/RNA ratio consequently increases parallel with age [23]. Generalizing their results, authors worked out a hypothesis, according to which in the course of ageing the sites for RNA synthesis sustain damages, where faulty messenger RNA will be formed and the latter produces enzymes, incapable of normal cell functions. Increase of RNA content could be explained by the supposition that accumulation of substrate molecules causes "de-repression" of RNA synthesis. Furthermore defective RNA was presumed to be less stable than the normal one: this leads to rise of the tracer uptake/RNA ratio. At present the theory consists of many details some of which are not proved; it would be highly interesting to know, for example, why the repressor is affected by the accumulation of substrate in one of the cell types, and does not alter the function in the other.

As main cause of faulty RNA production somatic-, or point mutations are supposed. Quantity of desoxyribonucleic acid itself does not decrease. Inversely, its concentration, calculated on wet or dry weight often increases. HRAHOVEC and ROCKSTEIN [14] observed this in muscles and in a minor degree in rat liver (the latter result was corroborated by DETWILER and DRAPER [9] and BÜRGER [5] in human brain). However the DNA/nucleus value, as demonstrated by FALZONE, BARROWS and SHOCK [10] does not modify even in old age. A contradiction arises here, because although polyploidy, increasing with age, can be made probable in liver, concerning other organs, e.g. brain, such explanation is impossible. Rise of DNA concentration in old age is not general; for example in case of acid desoxyribonuclease its activity decreases in bone marrow, remains on a constant level in thymus and spleen; only in the liver was an activity-increase of minor degree observed.

In greatest part of quantitative data (e.g. by analysis of whole organs) nucleic acid content of the parenchyma and interstitium, respectively is not separated. On the other hand, by more minute methods (e.g. cytophotometry) exclusively parenchymatous cells were examined. As in the course of ageing parenchyma and interstitium behave inversely (progress of the latter is striking), it would be desirable to know the age dependent alterations of the nucleic acid content of connective tissue-cells, as well. Elaboration of an information theory concerning ageing of the *whole organism* can be imagined only this way.

Contrary to other insect species, e.g. *Galleria* [1], *Drosophila* can be regarded without doubt as an ageing organism [6]. Constancy of RNA/DNA ratio is probably a resultant of modifications of opposite direction, similar to the above mentioned. We intend to supervise this postulate by cytophotometric analysis of parenchymal and interstitial cells.

The authors wish to acknowledge the excellent technical assistance of Mrs. Erzsébet Hollósi-Fazekas.

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ÄNDERUNGEN DES NUKLEINSÄURENGEHALTES VON *DROSOPHILA MELANOGASTER* IMAGOS IM VERLAUF DES REIFENS UND DES ALTERNS

Es wurde der RNS- bzw. DNS-Gehalt von weiblichen und männlichen *Drosophila melanogaster* Imagos in Abhängigkeit vom Lebensalter untersucht. Die Reifungsperiode der ♀♀ wird durch die Zunahme des Gesamtgewichtes, Trockengewichtes, DNS und RNS-Gehaltes charakterisiert. Die Synthese der Nukleinsäuren — besonders der DNS — übertrifft in dieser Periode die Zunahme aller anderen Komponenten und der RNS/DNS Quotient nimmt ab. Der DNS-Gehalt der ♂♂ nimmt ebenfalls zu, der RNS-Gehalt und damit der RNS/DNS Quotient wird geringer. Zu Beginn der Eiablage (6—12. Tag des Imago-Lebens) nimmt das Gesamt- und Trockengewicht und der Nukleinsäurengehalt zuerst ab und erreicht schließlich einen konstanten Wert. In der selben Periode findet man bei den ♂♂ eine geringfügige Abnahme von Gesamt- und Trockengewicht und des RNS-Gehaltes. Der DNS-Spiegel bleibt aber unverändert. Die Untersuchungen zeigen, daß für die erste, sog. Reifungsperiode bedeutende quantitative Veränderungen des genetischen Informationssystems charakteristisch sind. Im Altern sind mit der angewandten Methode keine quantitativen Änderungen der Nukleinsäuren nachzuweisen. Zur Erklärung dieses Befundes halten wir zwei Hypothesen für möglich: *a)* Im Altern spielen die quantitativen Änderungen der Nukleinsäuren neben den qualitativen, strukturellen Änderungen nur eine untergeordnete Rolle oder *b)* das Informationssystem ändert sich im Parenchym und im Interstitium im entgegegengesetzen Sinne und die quantitativen Änderungen gleichen sich aus. Zur Entscheidung dieser alternativen Möglichkeiten soll eine cytophotomerische Analyse der Organe von *Drosophila* angewandt werden.

ИЗМЕНЕНИЕ СОДЕРЖАНИЯ НУКЛЕИНОВЫХ КИСЛОТ ИМАГ ДРОЗОФИЛ *(DROSOPHILA MELANOGASTER)* В ПРОЦЕССЕ СОЗРЕВАНИЯ И СТАРЕНИЯ

Исследовали содержание РНК и ДНК имаг у мужских и женских особей дрозофил в зависимости от возраста. Период питания в процессе созревания и созревания яйцеклеток характеризуется увеличением сырого веса, веса сухого вещества, содержания РНК и ДНК у женских особей. Синтез нуклеиновых кислот, особенно ДНК, в этом периоде превышает синтез других компонентов тела, а соотношение РНК и ДНК снижается. У мужских особей также увеличивается содержание ДНК, а содержание РНК и вслед за ним и соотношение РНК и ДНК тоже уменьшаются. С наступлением откладки яиц (на 6—12 день имагинального развития) сырой вес, вес сухого вещества и содержание нуклеиновых кислот у женских особей уменьшаются в одинаковой степени, а позже приобретают постоянную величину. Незначительное снижение сырого веса, веса сухого вещества и содержания РНК у мужских особей наблюдаются и в дальнейшем, а содержание ДНК остается на постоянном уровне. В последнем периоде, — в периоде старения — соотношение РНК и ДНК имаг дрозофил остается без изменения, только лишь незначительное снижение содержания РНК наблюдается у женских особей. На основании наших исследований видно, что пока первый период, *период созревания*, характеризуется интенсивным количественным изменением информационной системы клеток, то в периоде старения количественные изменения нуклеиновых кислот нельзя выявлять с помощью применяемых нами методов. По нашим предположениям это можно объяснить двояко: (а) в периоде старения количественные изменения нуклеиновых кислот играют подчиненную роль по отношению качественных структурных изменений, или (б) информационная система преобразуется и количественно, но паренхима и интерстиции изменяются в противоположном направлении и таким образом изменения уравнивают друг друга. Для решения этих альтернатив в будущем приходится цитофотометрически проанализировать отдельные органы дрозофил.

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Acta Biol. Hung., **15** (3), 351—359 (1965)

PHOTOSYNTHESIS OF THE UNICELLULAR GREEN ALGAL STRAIN, SCENEDESMUS OBTUSIUSCULUS CHOD. AT VARIOUS pH VALUES

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(Received September 3, 1964)

Synopsis

Photosynthetic activity of the unicellular green algal strain 5618. *Scenedesmus obtusiusculus* CHODAT was measured by three different methods (Winkler's O_2, Warburg's manometric and Ruttner's conductometric methods) in the pH range between 4—11. Upper limit of photosynthesis was at pH 10.8, and a rather definite optimum was observable near pH 9.6. At pH 7 activity fell to the half of its maximum, and at pH 4.5 photosynthesis was equal to zero. The influence of the limited photosynthesis at low pH values on growth and the ammonium nitrate utilization of this strain are also discussed.

Introduction

In the mass culturing experiments with unicellular green algae started in 1959 at the Biological Research Institute of the Hungarian Academy of Sciences ammonium nitrate was used as nitrogen source [13]. The algal strain 5618. *Scenedesmus obtusiusculus* used in these studies preferred ammonium ions to nitrate ones.

As it is evidenced by some previous laboratory experiments, this strain does not utilize nitrate ions as long as ammonium salts are present in its nutrient medium. It was a plausible assumption that after the uptake of considerable proportion of ammonium ions the liquid phase of suspension turns acid which is promoted by the continuous feeding of CO_2. Therefore the antagonism between ammonium and hydrogen ions becomes gradually sharpened, the uptake of ammonium decreases and finally stops due to the competitive inhibition of hydrogen ions. Parallel with this, changes favourable for the uptake of nitrate ions may take place and the ionic balance of the solution becomes optimal with subsequent rise in the pH value.

Nevertheless, the correctness of the above assumption was not justified in practice. Because the amount of ammonium nitrate in the nutrient solution was rather high, 500 mg per litre, the pH of the suspension could not be restored, and nitrate uptake could not take place. It might be established by all means, that there were still ammonium ions left in the solution. At this advanced state of cultivation the pH was stagnant between 4.3—5.0. It was also very remarkable that there was a considerable increase in the crude protein content of the cell material up to about 70 per cent of dry matter. This inhibition of ammonium uptake was presumably due to the lack or deficiency of the amount of

assimilates i.e. carbohydrate or organic acid reserves in the cells resulted by the cessation of photosynthesis under these acid circumstances [5, 41].

According to some laboratory experiments which will be discussed elsewhere the ammonium uptake of healthy algal cells is not considerably inhibited at pH as low as 5.

It has been previously recorded by us that the strains isolated from the Lake Balaton and from the calcareous soils in the surroundings of Tihany are rather basophilous [11, 12]. The aim of the present paper is to discuss in detail the effect of hydrogen ion concentration on the photosynthetic activity in 5618. *Scenedesmus obtusiusculus* CHOD. over a wide range of pH values.

Material and method

The axenic algal strain used in these experiments: 5618. *Scenedesmus obtusiusculus* CHOD. (isolated in 1959 by ZSUZSA F. KALKÓ from an aquarium fed with Balaton lake water) is maintained on agar slants of the following composition: glass distilled water 1000 ml, potassium nitrate 1.0 g; magnesium sulphate cryst. 0.1 g; calcium nitrate 0.1 g; dipotassium phosphate 0.2 g; ferric citrate 0.002 g; citric acid 0.002 g; microelements in the solution of Arnon's A4 1 ml; glucose 5 g; peptone 2 g; yeast hydrolysate 2 ml; washed agar 15 g.

For preparatory cultures algae were scraped from agar slants and suspended into liquid medium having the following composition: Tihany tap water 1000 ml; ammonium nitrate 0.3 g; magnesium sulphate cryst. 0.1 g; potassium dihydrogen phosphate 0.25 g; ferrous sulphate cryst. 0.025 g; sodium ethylenediamin tetraacetate (EDTA) 0.01 g; Arnon's trace element solution A4 1 ml. By bubbling through this solution with 3 per cent carbon dioxide in air it arrives at equilibrium at pH 6.8—7.0.

Photosynthetic measurements were performed by three different methods.

The upper pH limit of photosynthesis was determined by Ruttner's [31, 32, 33] conductometric method. Suspensions containing about 1500 cells/μl [Bürker hemocytometer] were prepared of 2, 3 and 4 mM $KHCO_3$ solutions, and exposed in glass stoppered vessels at $25 \pm 0.1^\circ$ C in thermostate illuminated by a 300 W incandescent lamp (c. 6000 lux) cooled with water jacket. During the time of exposition the samples were shaken up several times. The rate of photosynthesis is determined by measuring the changes taking place in the physicochemical properties of $KHCO_3$ solutions. The measurements of electric conductance were made with the electronic instrument "Resistoscope" type HRE 13—58 (Híradástechnikai KTSz. Budapest) and with a platinum wire screen electrode of a capacity of C = 0.054. The carbon dioxide deficiency in proportion to the total amount of CO_2 present in the $KHCO_3$ solution was computed partly on the basis of Ruttner's nomographs [32] and partly on basis of the relation existing between pH and the carbonic acid components using Österlind's formulae [30].

A great many measurements were carried out by the usual Warburg manometric method [15]. The experimental solution used on this occasion was a 3 mM $KOH—K_2CO_3—KHCO_3$ buffer system prepared from aliquots of 3 mM KOH solution by bubbling washed pulmonary air through it. Solutions of known carbon dioxide content i.e. hydrogen ion concentration were adjusted by continuous electrical conductivity- or pH-measurements. For manometry 10 ml algal suspensions containing about 1 mg dry matter were used, in flat bottom vessels, illuminated by a 40 W fluorescent tube (Tungsram F2 "warm white" c. 7000 lux) built into the water bath, at a temperature of 25.0 ± 0.5 °C and at a shaking velocity of about 110 cycles/min.

The photosynthetic power at low pH-values was determined by Winkler's oxygen method. The experimental solution used was tap water enriched with NH_4NO_3. Its pH was adjusted by adding suitable amounts of 0.1 N nitric acid. Before preparing the experimental suspension (containing about 1000 cells/μl) the nutrient solution was evacuated for half an hour, then pure carbon dioxide was bubbled through it and was allowed to stand during one night. Next day the sufficient amount of algal cells were added. The suspension was poured into glass stoppered Maucha test tubes and exposed on a rotating shaking machine to an illumination of c. 7000 lux (produced by two F2 "warm white" luminescence tubes) for two hours

Oxygen content was measured by the usual Winkler method. Titration with 0.005 N thiosulphate solution was not disturbed by the small amount of cells.

The results obtained by the three methods are expressed in terms of μl O_2/1 mg dry matter/hour (Warburg's method), mg $O_2/10^9$ cells/hour (Winkler's method), mg CO_2/litre (Ruttner's conductometry). For the conversions the following factors were used: 10^9 cells equivalent to 54 mg dry matter; 1 mg CO_2 = 0.873 mg O_2 (PQ = O_2/CO_2 = 1.2); 1 mg O_2 = 700 μl; 1 mg CO_2 = 509 μl at NTP.

The pH measurements were made with a "pH-electrometer type 2512" (Orion-Budapest), and later with a "Beckman Model GS" portable pH-meter, with glass and calomel electrodes. Previous to every series of measurements the instrument was calibrated by Atkins-Pantin [3] and Beckman buffer solutions respectively.

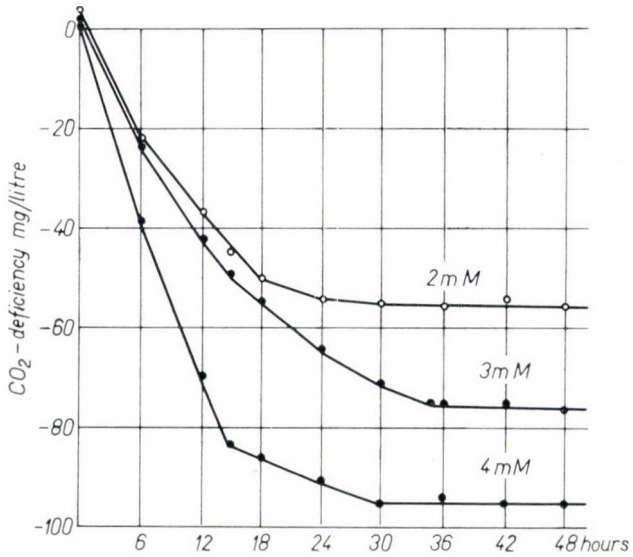

Fig. 1. Carbon dioxide uptake by *S. obtusiusculus* cells from the $KHCO_3$ solutions of three different concentrations

Results

The graphs (Fig. 1) illustrate the photosynthetic rates as changes in carbon dioxide deficit (mg/litre) in three differently concentrated $KHCO_3$ solutions measured conductometrically, in the function of time (hours). It is visible that at the 30—36th hour of exposition to light the decrease in the carbon dioxide content of the solutions due to the photosynthetic activity of the algal cells stops. In the three media examined photosynthesis ceases at different carbon dioxide (HCO_3 and CO_3) concentrations. The importance of pH in the milieu is also clearly evidenced by the results presented in Table 1. Photosynthesis stopped at pH 10.8—10.9 in all three media investigated. Because at these pH values the inorganic carbon sources of the solutions were not exhausted (the concentration of HCO_3 ions in 2 mM solution was 0.19 mM, and at the same time the 4 mM medium contained 0.42 mM $KHCO_3$), the hydrogen ion concentration may be regarded the main limiting factor of pho-

tosynthesis. On the other hand, the pH value between pH 10.8—10.9 may be taken for the upper limit of photosynthesis in the case of this strain.

Table 2 summarizes the results obtained by the above three methods. A rather definite optimum is observable between pH 9—10, and only about its half at pH 7. Photosynthesis ceases completely at pH 4.5.

The results were graphed in Fig. 2.

It must be noted that the results obtained by Ruttner's conductometry (half filled circles in Fig. 2) are charged with many factors of uncertainty in-

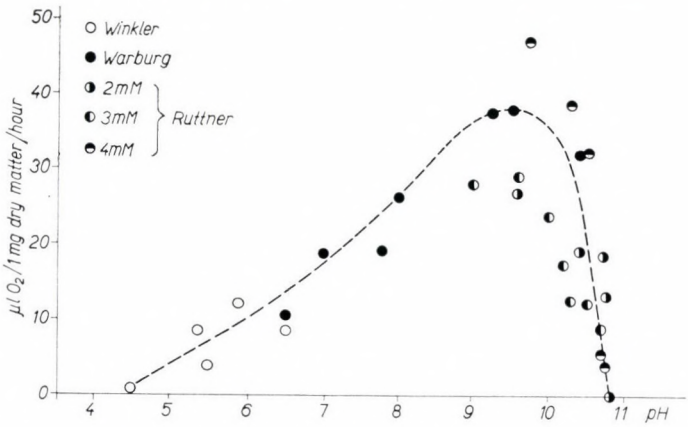

Fig. 2. Photosynthesis in *S. obtusiusculus* plotted against pH

herent in the method and due to the complicated conversion of mg CO_2/litre unit into μl O_2/1 mg dry matter.

Discussion

The basophilous character of this *Scenedesmus obtusiusculus* strain has been incontestably proved by the measurements presented above. This alga belongs to a group of our strains, that excels by rather strong resistance to high pH values. For the sake of comparison between the upper pH limits of

Table 1

Properties of three different media at the cessation of photosynthesis represented in Fig. 1

Concentration	$K_{18} \cdot 10^4$	CO_2-deficiency		$cHCO_3$ mM	cCO_3 mM	pH
		mg/litre	per cent			
2 mM	2.89	54.5	62.0	0.19	0.49	10.8
3 mM	4.08	76.0	57.6	0.28	1.02	10.9
4 mM	5.12	95.0	54.0	0.42	1.46	10.8

Table 2

Photosynthesis in Scenedesmus obtusiusculus at different pH values

pH	μl O_2/1 mg dry matter per hour	Method
4.5	0.6	Winkler
5.4	8.7	,,
5.5	3.9	,,
5.9	11.9	,,
6.5	8.6	,,
6.5	10.8	Warburg
7.0	19.0	,,
7.8	19.0	,,
8.0	26.4	,,
9.0	28.1	Ruttner 3 mM
9.3	37.8	Warburg
9.5	38.0	,,
9.6	27.0	Ruttner 2 mM
9.6	29.0	,, 3 mM
9.8	49.0	,, 4 mM
10.0	23.8	,, 3 mM
10.2	17.7	,, 3 mM
10.3	12.6	,, 3 mM
10.3	38.9	,, 4 mM
10.4	19.4	,, 2 mM
10.4	32.0	Warburg
10.5	12.6	Ruttner 3 mM
10.5	32.3	,, 4 mM
10.6	12.6	,, 3 mM
10.6	8.8	,, 4 mM
10.7	13.8	,, 2 mM
10.7	8.8	,, 3 mM
10.7	5.6	,, 4 mM
10.75	4.9	,, 4 mM
10.8	0.0	,, 2 and 4 mM
(10.9)	0.0	,, 3 mM

photosynthesis in different strains of our collection the following list is given [9, 10, 12].

		upper pH limit
7k	*Chlorella vulgaris* BEYER	9.5
4061	*Scenedesmus quadricauda* (TURP.)BRÉB.	10.3
5618	*S. obtusiusculus* CHOD.	10.8
516	*Kirchneriella sp.*	11.1
198	*Scenedesmus quadricauda* (TURP.) BRÉB.	11.2
4069	*S. tenuispina* CHOD.	11.2
3153	*Chlorocloster terrestris* PASCHER	11.3
4063	*Scenedesmus armatus* CHOD. var. *boglariensis* HORTOB.	11.3
953	*Coelastrum microporum* NÄG.	11.4
525	*Scenedesmus acuminatus* (LAGERH.) CHOD. var. *falcatus* CHOD.	11.4
636	*S. armatus* CHOD.	11.4
4070	*S. spinosus* CHOD.	11.5

It has been suggested [42] that the pH of soils does not play an important role in the photosynthetic activity of land-plants; on the other hand, it has been demonstrated by ELBERTZHAGEN and KANDLER [7] that the rate of the photosynthetic fixation of carbon-dioxide of the chloroplasts is positively influenced by the hydrogen ion concentration within the leaf cells.

The situation is different in the case of submerged aquatic plants, especially of unicellular algae. The experimental establishment of pH-effect on growth and nutrition is not simple, because the hydrogen ion concentration of the milieu surrounding the cells may considerably change in the course of culturing, and the correct interpretation of the results is difficult, because it is frequently impossible to isolate the direct effects of hydrogen ion concentration from other contributing factors (differential absorption of various ions, exhaustion of carbon dioxide from carbonic acid system, accumulation of some metabolites in the medium, precipitation of some salts and elements at extreme pH values etc.). Nevertheless, most physiological works on algae dealing with the effect of pH are engaged in similar problems. There are literary data emphasizing the unusually high resistance of euglenoid flagellates at acid solutions [16, 21] but the preference to basophilic circumstances of blue-green algae has been proved by many culturing experiments [44, 14, 26, 43, 46]. Likewise, the alga *Dunaliella euchlora* [25] and other marine algae [22, 17, 4] display a better growth at pH values above 7. On the other hand, most fresh-water algae examined from this point of view cannot tolerate high pH values, for they require free carbon dioxide for their photosynthesis [18, 19, 24, 17, 27, 45].

Quite a number of authors [8, 35, 36, 29, 30, 6, 24, 12] investigated the relationship between the photosynthesis of unicellular fresh-water algae and pH. The experimental data obtained by EMERSON and GREEN [8] represent an important contribution to our knowledge on the influence of pH on *Chlorella* photosynthesis. It was observed by these authors that the rate of photosynthesis is constant in the pH-range between 4.6—8.9 if both carbon dioxide and light were saturated. Under certain circumstances a decreasing effect of low pH on photosynthetic activity in saturated light in *Chlorella* may be found [39]. Normally, however, the rate of photosynthesis in algae that are unable to utilize hydrocarbonate ions is independent of pH [38, 40, 34]. An exceptionally highly acid-resistant *Chlorella ellipsoidea* strain was recorded by KESSLER and KRAMER [23]. ALLEN [1] has found a *Cyanidium* strain, able to grow in normal sulphuric acid solution [20]. Most authors are of the opinion that in the life of submerged aquatics of this type pH may effect photosynthesis indirectly by changing the amount of available carbon dioxide [42, 37, etc.].

In view of the results obtained by experiments with algal strains able to utilize hydrocarbonates [30, 17, 11, 12] two things must be emphasized. First, upon the evidence of previous experiments [12] and results represented here not the quantity of the inorganic carbon form present in the system but the pH seems to be the most significant limiting factor of hydrocarbonate absorption in the case of photosynthesis in $KOH—K_2CO_3—KHCO_3$ buffer solutions of various molar concentrations. Secondly, it deserves attention that there is a more or less definite pH optimum in the intensity of photosynthetic activity in this latter type of unicellular algae.

In order to get an answer to the problem of ammonium nitrate utilization data in Table 2 must be considered. It is seen from this table that there is a decrease in photosynthetic activity to the half and to the quarter of the optimal value below pH 7 and about pH 6 respectively.

It is assumable therefore, that the adsorption of ammonium ions is due besides the hydrogen-ion antagonism developing by the acidifying of solution also to quantitative or qualitative [28] changes in the composition of photosynthates. For more detailed knowledge of the effect of pH in the utilization of various nitrogen sources further investigations are needed.

Grateful acknowledgement is made to Mrs. ZSUZSA F. KALKÓ for helpful assistance during this work, and to Mr. F. LUKACSOVICS for his valuable advices on some computation problems.

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UNTERSUCHUNGEN ÜBER DIE PHOTOSYNTHESE DES EINZELLIGEN GRÜNALGEN-STAMMES *SCENEDESMUS OBTUSIUSCULUS* CHOD. BEI VERSCHIEDENEN pH-WERTEN

Die photosynthetische Aktivität des einzelligen Grünalgen-Stammes 5618. *Scenedesmus obtusiusculus* CHODAT wurde im pH Bereich 4—11 mit drei verschiedenen Methoden (Winkler's O_2, Warburg's manometrische und Ruttner's konduktometrische Methode) untersucht. Die obere Grenze der Photosynthese war bei pH 10,8 und ein ausgesprochenes Optimum annähernd bei pH 9,6 feststellbar. Bei pH 7 ging die Aktivität auf die Hälfte ihres Maximums zurück und bei pH 4,5 hörte die Photosynthese ganz auf. Es wird die Bedeutung der bei niedrigen pH-Werten eingeschränkten Photosynthese auf das Wachstum und auf die Ammoniumnitrat-Verwertung dieses Stammes besprochen.

ФОТОСИНТЕЗ ОДНОКЛЕТОЧНОЙ ЗЕЛЕНОЙ ВОДОРОСЛИ *SCENEDESMUS OBTUSIUSCULUS* CHOD. ПРИ РАЗЛИЧНОМ ЗНАЧЕНИИ pH

Фотосинтетическая деятельность штамма № 5618 одноклеточной зеленой водоросли *Scenedesmus obtusiusculus* CHODAT изучалась тремя различными методами (метод определения O_2 по Винклеру, манометрический метод Варбурга и кондуктометрический метод Руттнера) в пределах pH = 4—11. Верхний предел фотосинтеза наблюдался при pH = = 10,8, а довольно хорошо выраженный оптимум — вблизи pH = 9,6. Активность фотосинтеза при pH = 7 снизилась на половину, а при pH = 4,5 была равна нулю. Влияние пониженного при низких значениях pH фотосинтеза на рост и поглощение азотнокислого аммония обсуждается также на примере указанного штамма.

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Acta Biol. Hung., **15** (3), 361—369 (1965)

EFFECT OF STRIGA HERMONTHICA BENTH. AND NITROGEN APPLICATION ON THE GROWTH AND NITROGEN CONTENT OF SORGHUM VULGARE LUR.

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(Received October 3, 1964)

Synopsis

Sorghum was grown in pots to assess the effects of nitrogen application on dry matter and nitrogen yields of both *Striga hermonthica* and forage sorghum. Nitrogen application significantly increased dry-matter yield of sorghum irrespective of *Striga* attack. The incidence of S. *hermonthica* was, however, not significantly affected by fertilizer application. *Striga* parasitism on sorghum resulted in an average loss of 63.8% in dry weight of shoots. The yield of nitrogen in sorghum shoots was also reduced by 54.3%. Dry weight and nitrogen content of roots were not affected by *Striga*. *Striga* contributed 10.5% of the dry matter produced, but it recovered 24.9% of the total nitrogen yield. The nitrogen concentration in *Striga* was three times as much as that of the host plant and was not significantly affected by nitrogen application.

Introduction

Striga hermonthica, the common witchweed, parasitizes a wide range of tropical crops causing great losses in yields. The seeds of this semi-parasite germinate when stimulated by root exudates from both host and non-host plants [2]. The nature of such exudates is not yet identified, although D-xylo-ketose has been used to stimulate a small percentage of *Striga* seeds [4]. Germination stimulants are synthesized by actively growing cells in the cell elongation zone of host roots and are excreted as the cells are completing their elongation [11]. After germination, *Striga* seedlings send out haustoria which make contact with the stele of the host root [12]. When this contact is made, green aerial shoots of the parasite may be produced.

Sorghum vulgare is particularly vulnerable to witchweed attack when grown in clay soils in Northern Sudan. Severe reduction in sorghum grain yields have been reported by many workers [2, 8, 9, 10, 13]. In potted sorghum, ANDREWS [3] has found that *S. hermonthica* attack resulted in heavy reduction in dry matter of both roots and shoots of the host.

Fertilizer nitrogen was found to reduce *S. hermonthica* attack and improve grain yields of some grain sorghum varieties [3, 8]. However, LAST [8], has reported that nitrogenous fertilizer given to sorghum variety Dibekri never decreased the amount of *S. hermonthica* significantly and often increased it. DOGGET [6, 7] found that fewer aerial shoots of *S. hermonthica* developed on the sorghum variety Dobbs than on other types. It is obvious, therefore, that

the value of fertilizer nitrogen as a control measure against *S. hermonthica* infestations depends on the sorghum variety, but the mechanism involved in the control of *S. hermonthica* by nitrogen application is still obscure. It has been shown that sorghum resists infestation when conditions favouring growth are improved by the addition of nitrogenous fertilizer. Such additions of nitrogen also restrict development of the aerial shoots of the parasite [8].

In the Sudan, research work on the control of *S. hermonthica* was carried out with grain sorghums, while no attention was given to forage varieties. Recently, the production of fodder sorghum is taking a prominent place in dairy farming in the irrigated areas of Northern Sudan. It is the object of this paper to provide information about the effect of nitrogen application on dry matter and nitrogen content of both *S. hermonthica* and forage sorghum when grown in pots.

Material and method

The experiment was carried out in the Demonstration Farm of the Faculty of Agriculture, Shambat, during 1961. Clay pots, 25 cm in diameter containing 5 kg of Nile silty soils were used. The experiment was laid down as a random block with 12 replicates. Four levels of nitrogen in the form of ammonium sulphate (21% N), viz: 0, 40, 80 and 120 lb. N per feddan were applied on area basis to both infested and uninfested pots. Infestation of pots was made by thoroughly mixing 0.5 g of *S. hermonthica* seeds with the soil in each pot. Eight seeds of *Sorghum vulgare* var. Abu Sabein were sown in each pot in October 1961. Soon after germination *Sorghum* plants were thinned to 5 plants per pot.

Twelve weeks after sowing, sorghum shoots were cut at soil surface and the contents of each pot were washed out. The plant material was then dried in an oven at 70 °C to a constant dry weight, which was recorded.

Nitrogen analyses were made on the oven-dry samples using the conventional Kjeldahl oxidation followed by Markham semi-micro distillation.

Results

Incidence and dry matter yield of Striga hermonthica

Nitrogenous fertilizer had no significant effect on the total number of emerged *Striga* shoots nor on the flowering of these shoots. The average total numbers of *Striga* shoots per pot were 14.7 at N = 0, 10.0 at N = 40 lb., 9.3 at N = 80 lb. and 12.3 at N = 120 lb. per feddan.*

The addition of ammonium sulphate tended to reduce dry matter produced by *S. hermonthica* (Table 1). This tendency, however, did not reach significant levels.

Dry-matter yield of Sorghum vulgare

Effect of nitrogen fertilizer

Applied nitrogen resulted in a highly significant increase in shoot dry matter irrespective of *S. hermonthica* attack. On the other hand, dry matter

* 1 feddan = 0.42 hectares.

production by sorghum roots showed an insignificant rise following fertilizer application.

Effect of *Striga hermonthica* on *Sorghum vulgare*

Fig. 1 shows that *Striga* attack resulted in severe stunting and inhibition of the growth of sorghum plants.

Fig. 1. The effect of parasitism by *Striga hermonthica* on the growth of *Sorghum vulgare* afte 12 weeks.

N_0, N_1, N_2, N_3 indicate levels of nitrogen equivalent to 0.0, 40, 80 and 120 lb. N per feddan, respectively. B: indicates the presence of *Striga*

In the control pots the effect was so severe that death of the host shoots took place 7 weeks after *Striga* emergence.

Table 1 shows that *Striga* attack resulted in 53.3% reduction in dry matter produced by the sorghum plant. The dry weight of sorghum roots maintained a remarkable uniformity in both parasitized and unparasitized plants. The effect of *Striga hermonthica* was thus mainly reflected on sorghum shoots whose dry matter was decreased by 63.8%.

It may also be noted that the total dry-matter production per pot was significantly greater in the uninfested pots, i.e. sorghum alone, than in pots infested with the parasite. This observation clearly indicates that the unparasitized host is more efficient in building up dry matter than the host and parasite when grown together.

Table 1

Dry-matter yields of Sorghum and Striga when fertilized with different rates of nitrogen (calculated as g dry matter/pot)

Treatment	Dry-matter yield of				Total dry-matter yield per pot
	Sorghum root	Sorghum shoot	Root& shoot	*Striga*	
N_0B_0	7.4	32.9	40.3	—	40.3
N_0B_1	7.9	8.3	16.2	4.5	20.7
N_1B_0	9.7	41.3	51.0	—	51.0
N_1B_1	8.5	15.2	23.7	3.0	26.7
N_2B_0	8.7	47.4	56.1	—	56.1
N_2B_1	11.1	22.8	33.9	1.9	35.8
N_3B_0	10.6	62.2	72.8	—	72.8
N_2B_1	8.9	20.0	28.9	2.7	31.6
Effect of increasing level of supplied fertilizer on dry-matter yield					
N_0	7.7	20.6	28.3	4.5	30.5
N_1	9.1	28.3	37.4	3.0	38.9
N_2	9.9	35.1	45.0	1.9	46.0
N_3	9.8	41.1	50.9	2.7	52.2
L. S. D. at P= 0.05		7.30	8.83		9.11
0.01		9.76	11.82		12.20
Effect of Striga attack on dry-matter yield					
No *Striga*	9.1	45.9**	55.0**	—	55.0**
Striga	9.1	16.6	25.7	3.0	28.7
% reduction in total dry matter		63.8	53.3		47.8

** = Highly significant
N_0 = no nitrogen; N_1 = 40 lb. N per feddan; N_2 = 80 lb. N per feddan; N_3 = 120 lb. N per feddan
B_0 = uninfested; B_1 = infested

Nitrogen content of Striga hermonthica and Sorghum vulgare

Results of the nitrogen analyses are recorded in Table 2 and Fig. 2. The main points are:

(1) Fertilizer application tended to raise the concentration of nitrogen in the parasite tissues. The average nitrogen content of *S. hermonthica* grown in pots was 19.8 mg per gramme of dry matter, a concentration which is more

Table 2

Yields of nitrogen in Sorghum and Striga when fertilized with different rates of nitrogen (calculated as mg total N/pot)

Treatment	Nitrogen content of				Total yield of nitrogen per pot
	Sorghum root	Sorghum shoot	Root & shoot	*Striga*	
N_0B_0	39.7	133.9	173.5	—	173.5
N_0B_1	52.0	47.7	99.7	63.6	163.3
N_1B_0	54.3	189.7	244.0	—	244.0
N_1B_1	56.0	104.3	160.3	64.3	224.6
N_2B_0	53.0	245.8	299.2	—	299.2
N_2B_1	75.2	131.6	206.8	36.6	243.4
N_3B_0	68.9	310.7	379.6	—	379.6
N_3B_1	62.2	118.6	180.8	49.7	230.5
Effect of increasing level of applied fertilizer on yield of nitrogen					
N_0	45.8	90.8	136.6	63.6	168.4
N_1	55.2	147.0	202.0	64.3	234.3
N_2	64.3	188.7	253.0	36.6	271.3
N_3	65.6	214.7	280.2	49.7	305.1
L. S. D. at P = 0.05		50.2	63.1		67.5
= 0.01		67.2	84.4		90.4
Effect of Striga attack on yield of nitrogen					
No *Striga*	54.1	220.0	274.1**	—	274.1**
Striga	61.4	100.5	161.9	53.6	215.5
% reduction in total nitrogen		54.3	40.9		21.4

** = Highly significant
N_0 = no nitrogen; N_1 = 40 lb. N per feddan; N_2 = 80 lb. per feddan
N_3 = 120 lb. N per feddan. B_0 = uninfested; B_1 = infested

than three times that of the host root or shoot. Although statistical analysis of the nitrogen content in *S. hermonthica* did not show significant differences between different nitrogen treatments, yet it is clear from Fig. 2 that with fertilizer addition equivalent to 40 lb. N per feddan the nitrogen concentration showed an increase of 50% over the unfertilized control and remained almost unchanged with higher fertilizer applications. This observation may suggest a competition for nitrogen in which the host is more successful at levels of nitrogen higher than 40 lb. per feddan.

(2) The total nitrogen recovered by *S. hermonthica* tended to decrease slightly with increased fertilizer application. This is mainly a reflection of the observed decrease in *Striga* dry matter accrueing from nitrogen application.

(3) Fertilizer application resulted in insignificant variations in the concentration of nitrogen in sorghum roots and shoots.

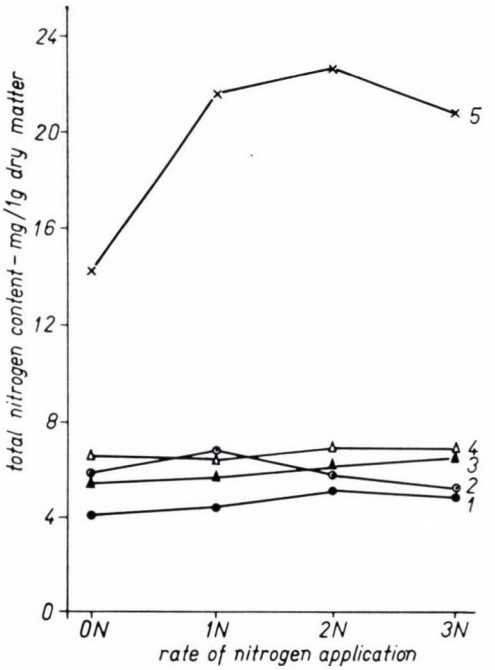

Fig. 2. Effect of fertilizer application on the nitrogen content (mg N/1 g dry weight) of infested and non-infested *Sorghum* shoots and roots and *Striga*

1 = uninfested *Sorghum* shoot
2 = infested *Sorghum* shoot
3 = uninfested *Sorghum* root
4 = infested *Sorghum* root
5 = *Striga*

(4) Infested sorghum roots and shoots contained significantly more nitrogen per gramme of dry matter than the corresponding parts of uninfested sorghum. Such accumulation of nitrogen is expected in view of the stunted growth and reduced host dry matter resulting from *S. hermonthica* attack.

(5) The total nitrogen recovered by the sorghum plant as a whole rose significantly with fertilizer application irrespective of *Striga* attack. However, this rise was mainly centred in sorghum shoots whose analysis of variance revealed a highly significant difference while there was only a slight increase in nitrogen recovered by roots.

(6) *S. hermonthica* attack caused 40.9% in the total amount of nitrogen recovered in the sorghum plant. It may be noted, however, that, while shoot nitrogen was reduced by 54.3%, the total nitrogen recovered by the host roots actually showed a slight increase as a result of *S. hermonthica* parasitism.

Although the parasite constituted only 10.5% of the total dry matter produced per pot, it recovered 24.9% of the total nitrogen. When only the aboveground parts are considered, the parasite contributed 15.3% of the dry matter but made up 34.8% of the total nitrogen.

Discussion

When grown in pots, the incidence and growth of *Striga hermonthica*, judged by number of shoots, dry matter production and nitrogen content, were not significantly influenced by addition of nitrogenous fertilizer. In field experiments *S. hermonthica* incidence was found to be greatly reduced by nitrogenous manuring [1, 5, 8, 9, 10]. In this investigation, the host roots were pot-bound at all levels of nitrogen application and this may have given the parasite similar subterranean conditions as a result of restricted root growth. Also the pots were rather widely spaced and consequently the shading effect of the host plant on the semi-parasite has been eliminated.

The growth of the sorghum plants was significantly improved by the use of nitrogenous fertilizer irrespective of *S. hermonthica* attack. The increase in dry-matter production by infested sorghum with the application of nitrogen may be partly attributed to the observed, although not statistically significant, decrease in the incidence of the parasite. These results, which have been reported by earlier investigators working with irrigated grain sorghum [5, 8, 9, 10] form the basis of the current use of nitrogenous fertilizers as a means of improving yields of *Striga*-infested or uninfested sorghum crops.

Production of dry matter by the sorghum plants as a whole was severely impaired by *S. hermonthica* attack. This effect was very pronounced in the shoots whose dry weight was reduced by 63.8% while the roots' dry weight showed a remarkable similarity in both infested and uninfested pots. ANDREWS [2] reported a reduction of 67% in both root and leaf weight as a result of *S. hermonthica* attack. The discrepancy in the results of the two experiments may be attributed to the effect of the different durations of the two experiments. In ANDREWS' experiment the roots were washed out 6 weeks after sowing. *Striga* seedlings were found attached to sorghum roots 7 days after sowing but it was only in the fifth week after sowing that any *Striga* emerged above soil surface. During this period the infestation developed very quickly below soil surface. As the parasite at the underground stage was completely dependent on the host plant, the sorghum plant was severely depleted of its food material and consequently growth of both root and shoot was restricted. In the present

investigation, *Striga* emerged 5 weeks after sowing and the roots were washed out 7 weeks later. No differences in root dry weight were detected between the parasitized and non-parasitized sorghum plants. It may, therefore, be postulated that by the emergence of *Striga* aerial shoots the dependence of the parasite on the host was partially released to the extent which did not affect the root growth. Furthermore, the sorghum roots in this experiment were found pot-bound under all treatments, a condition which, undoubtedly, greatly influences normal root development and thus obliterates the true picture of the dry weight of root.

Although *S. hermonthica* caused a reduction of 53.3% in sorghum dry weight, it only contributed 10.5% of the total dry-matter production. Considering the aboveground parts alone, the parasite made up 15.3% of the total dry weight but it caused a reduction of 63.8% in the host-shoot dry-matter. It is, therefore, obvious that the parasite seriously impairs the capacity of the forage plant to build up dry matter. Even more serious is the effect of infestation on nitrogen yield of sorghum. *Striga* attack caused a reduction of 54.3% in nitrogen yield of the host shoots. The total nitrogen recovered per pot was reduced by 21.4%. This reduction in nitrogen uptake indicates that uninfested sorghum plants are more efficient in nitrogen utilization than infested sorghum and parasite when grown together.

S. hermonthica constituted only 10.5% of the dry matter produced per pot, but it recovered 24.9% of the total nitrogen. When only the aboveground parts are considered the parasite made up 15.3% of dry matter and contributed 34.8% of the total nitrogen. This relatively high contribution in yield of nitrogen compared to dry-matter production is due to the high concentration of nitrogen in the parasite tissues which averaged three times that of the host plant.

The results of this investigation clearly show that *Striga* parasitism has a most unfavourable effect on dry matter and nitrogen yields of fodder sorghum. The parasite not only reduces the efficiency of the host to use nitrogen but it also retained a high proportion of the total absorbed nitrogen in its tissues which are not readily eaten by farm animals.

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ÜBER DIE WIRKUNG VON *STRIGA HERMONTHICA* BENTH. UND STICKSTOFFZUFUHR AUF DAS WACHSTUM UND DEN STICKSTOFFGEHALT VON *SORGHUM VULGARE* LUR.

Sorghum-Pflanzen wurden in Blumentöpfen gezüchtet um die Wirkung von Stickstoffzugaben auf das Trockengewicht und den Stickstoffgehalt sowohl von *Striga hermonthica* als auch von Sorghum zu untersuchen. Die Nitrogenzufuhr erhöhte das Trockengewicht von Sorghum signifikant, unabhängig vom Striga-Befall. Das Vorkommen von *Striga hermonthica* wurde vom Stickstoffdünger nicht signifikant beeinflußt. Striga-Parasiten verursachten eine Abnahme des Trockengewichtes des Sprosses von Sorghum um durchschnittlich 63,8%. Auch der Nitrogengehalt des Sorghum-Sprosses wurde um 54,3% vermindert. Trockengewicht und Nitrogengehalt der Wurzeln wurden vom Parasitenbefall nicht beeinflußt. *Striga* gab 10,5% der ganzen gebildeten Trockensubstanz, verbrauchte aber 24,9% der gesamten Nitrogenmenge. Die Nitrogenkonzentration betrug in *Striga* das dreifache der Konzentration in der Wirtspflanze und diese Konzentration wurde von der Stickstoffdüngung nicht signifikant beeinflußt.

ЭФФЕКТ *STRIGA HERMONTHICA* BENTH. И АПЛИКАЦИИ АЗОТА НА РАЗВИТИЕ И СОДЕРЖАНИЕ АЗОТА В *SORGHUM VULGARE* LUR.

Авторы выращивали сорго в горшках, чтобы наблюдать влияние апликации азота на сухое вещество и содержание азота в *Striga hermonthica* и кормовом сорго. Апликация азота значительно повысила содержание сухого вещества сорго, несмотря на вредное влияние паразита *Striga*. На наличие паразита *Striga hermonthica* искусственным удобрением нельзя было эффективно влиять. В результате паразитизма *Striga* средняя потеря сухого вещества стебля сорго была 63,8%. Содержание азота в стеблях сорго понизилось также на 54,3%. *Striga* не оказала никакого вредного влияния на содержание сухого вещества и азота в корке растения. *Striga* составляет 10,5% врыабатываемого сухого вещества, а 24,2% всего содержания азота. Концентрация азота в паразите *Striga* была три раза больше, чем в растении-хозяине и не была значительно изменена применением азота.

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RECENSIONES

Problems in Biology. (Vol. 1)

Edited by G. A. KERKUT

Pergamon Press, Oxford, London, New York, Paris (1963) 223 pp., 42 s.

The international literature on comparative animal physiology is not very rich in textbooks, monographs or even in up-to-date reviews on the actual problems of this science. This is why the presentation of the series *Problems in Biology* by the Pergamon Press is a very useful and promising initiative.

The first volume of this series, edited by the well known physiologist G. A. KERKUT deals with two main problems: the function of the respiratory pigments of invertebrates and the bioelectric potentials.

The chapter on the respiratory pigments of invertebrates was written by J. D. JONES of the University of Sheffield. It presents a very clear and comprehensive review on the different respiratory body-fluid-pigments in lower animals beginning with the chlorocruorin and the haemocyanin, and finishing with the several kinds of haemoglobin. The second chapter on bioelectric potentials written by M. A. P. RIDGE and R. J. WALKER of the University of Southampton has two parts: the first on steady potentials, the second on rapid changes, i.e. action potentials.

These latter two articles have a particular value for the physiologists working in other fields than that discussed in this chapter. The essay gives a very clear account on the present state of the different theories of the resting and action potential. The selective method of the authors, their personal approach ensures a highly logic argumentation, a very obvious point of view.

The glossary appended to the last chapter explaining some of the terms is useful for those not familiar with physical and physico-chemical terminology.

The carefully got-up volume praises the excellent work of the Pergamon Press.

GYÖRGY ÁDÁM (Budapest)

F. DESSAUER:

Quantenbiologie

2nd edition, edited and completed by K. SOMMERMEYER, Springer-Verlag, Berlin, Göttingen, Heidelberg (1964), 286 pp., 39.60 DM.

1922, the year when the hit theory of nuclear biological effects has been published by DESSAUER for the first time constitutes a landmark in the history of nuclear genetics and nuclear biology in general. In the period that followed, DESSAUER's co-workers including SOMMERMEYER, the editor of the present book and RAJEWSKI, the author of its introductory part, worked out the mathematical formulation of this theory, expanded it to what came to be termed as target theory and developed it with the indirect hit theory.

The political changes which started in 1933 forced DESSAUER to go into exile and this made it difficult for him to take a personal part in the improvement of his theory. It became only possible for him after the Second World War to give in the first edition of a book of extremely short extent a summary of the theses of quantum biology, their mutational genetical consequences and some related new problems such as the question of the inactivation of viruses and phages, etc.

At the time when the summary was published our knowledge of the nucleic acids was much less than today. It is an indication of DESSAUER's greatness that in spite of the extension

of knowledge his conclusions remain basically correct even today. It is easy to understand that when DESSAUER died in 1963 and K. SOMMERMEYER, one of his closest co-workers, was invited by the publisher to prepare the new edition for publication, he adopted the method of leaving the text of the original work unchanged, and adding a second part on the present state of quantum biology.

The part written by SOMMERMEYER discusses three major problems. The first section deals with the general problems of hit theory, namely the analysis of dosis effect curves and the primary processes are clarified on this basis. The application of hit theory for the explanation of the inactivation of enzymes, viruses and bacteria and for their mutational processes is described in the second section, and finally in what appears to be an appendix the effects of perceptible light on photosynthesis and on the mechanism of sight are discussed.

In the introduction of the book, which may be considered an excellent piece in terms of scientific biography, RAJEWSKI pays tribute to DESSAUER.

The book is highly exciting not only because of its content but also because of its richness in ideas; it is very modern and what leaves the reader with something to remember for long is, above all, its extremely strict and logical argumentation. As a convenience for readers unfamiliar with biophysics both DESSAUER and SOMMERMEYER use a relatively small and not too complicated mathematical apparatus for expounding their ideas.

Finally, it is certain that for everyone interested in the methodological foundations of natural sciences DESSAUER's short essay on the application of thoughts on physics and mathematical formalisms in biology, which extend to some ten pages and is given as an appendix, will be of particular appeal.

This book is equally useful for biologists, physicists and chemists and for those who take an interest in the history of sciences and epistemology.

The traditional attractive typography of the publishers is a major asset which helps to read the book with ease.

TAMÁS ÁCS (Budapest)

A. ÁBRAHÁM:

Die mikroskopische Innervation des Herzens und der Blutgefässe von Vertebraten

(Microscopic innervation of the heart and blood vessels of vertebrates)

Akadémiai Kiadó, Budapest (1964), 458 pp., 222 illustrations

In the present monograph Professor ÁBRAHÁM summarizes investigations from his scientific activities of several decades dealing with cardial and vascular innervation. The first short chapter of the book gives a detailed description of the neurohistological methods employed which proved to be most advantageous, including his modification of Bielschowsky's method. The next and largest chapter treats the innervation of the heart. This highly didactic part discusses the problem both in the vertical and horizontal aspects. A phylogenetic view is throughout maintained; the cardial innervation of fishes, amphibians, reptiles, avians and mammals is described in seperate sub-chapters. At the same time the innervation of the different parts of the heart is also discussed in detail within each group (sinus venosus, bulbus arteriosus, ventricles, atria and crus of the atrioventricular bundle, and structure of the cardial ganglia). The necessary comparative anatomical relationships of the heart itself are discussed fully in the related chapters. Different types of effector nerve terminals are described in detail. It should be regarded as a special merit that apart from traditional neurohistological techniques the work informs also on examinations concerning the localization of acetylcholinesterase. In the next (3rd) chapter the receptors (epicardium, myocardium, endocardium) of the heart are discussed. Author established with the degeneration method that in their majority the afferent fibers of the heart are of vagus origin, some of them, however, are connected with the first thoracic spinal ganglia.

In the chapter on the innervation of the blood vessels special attention is given to the innervation of the layers of the vascular walls, to the structure of the pressoreceptors, carotid sinus and the innervation of the main arteries. Observations made on human pathological material (aorta, carotid sinus) are of special interest. The last chapter deals with the structure of chemoreceptors and describes the innervation of the arteries of the heart, kidney and central nervous system.

The book supplies a great deficiency, long felt not only by theoretical neurobiologists but with regard to its wide range of human experimental material also by clinicians who will

find it most useful in medical practice. To those, who are interested in the ultrastructural examination of the heart, vessels and in general of the vegetative nervous system, it will be of special importance, serving as a compass: It should under all circumstances be borne in mind that the main prerequisite of electron microscopic, ultrastructural studies of the nervous system is the perfect knowledge of the material on the light microscopic level.

The composition of the book, its manner of treating the subject is adequate. It might have been advantageous — for a more convenient orientation — to insert short summaries following the larger chapters. An extensive bibliography including 479 references completes the work.

The tasteful execution of the book and its beautiful illustrations do credit to the publishers.

JÓZSEF HÁMORI (Budapest)

Symposia Biologica Hungarica (Vol. 3) Regeneration and wound healing Symposium in Budapest, November 1960

Edited by GY. SZÁNTÓ

Akadémiai Kiadó, Budapest (1964), 148 pp.

Bulgarian, Hungarian, Polish and Russian scientists (biochemists, biologists, surgeons — experimental workers and clinicians) assisted at this Symposium. Their lectures delivered as well as the related discussions are presented in this volume.

Main subject of the Symposium was a manysided discussion of the biological and biochemical mechanisms underlying wound healing; some lectures, however, have dealt with general problems of regeneration and somatic embryogenesis, connections between regeneration and development of tumours, etc.

The lecture of B. P. TOKIN on "Regeneration and somatic embryogenesis" (pp. 11—45) has summarized, essentially, the main problems dealt with in his well known book (published in Russian under the same title), that is the most general problems concerning regeneration and related phenomena, first of all, that of somatic embryogenesis. Main problems involved are: classification of the regeneration processes; differences between regeneration and somatic embryogenesis; interrelations between integration level and asexual reproduction and somatic embryogenesis; interrelations between regenerative capacity and somatic embryogenesis within the systematical categories; regeneration and ontogenesis; evolution of somatic embryogenesis and regeneration.

Other lectures have dealt with various important related problems in connection with concrete experimental works or with clinical investigations. They are entitled in the volume as follows:

J. NIVELINSKI: "Activity of intracellular enzymes in regenerating new limbs" (pp. 47—53).

R. TSANEV: "Role of nucleic acids in the wound healing process" (pp. 55—73).

B. KELLNER and J. SUGÁR: "Comparative studies on the dynamics of regeneration in the course of wound healing and growth of skin cancer" (pp. 75—80).

J. SUGÁR and B. KELLNER: "Comparative studies on the tonofibrillar structure in the course of wound healing and skin carcinogenesis" (pp. 85—91).

I. KROMPECHER and L. SZODORAY: "Data to the histochemistry and biochemistry of ulcus cruris (varicose ulcer)" (pp. 93—98).

G. SZÁNTÓ, O. SZÉKELY and SUSANNA SZŐNYI: "Regional differences (axial gradients) in wound healing" (pp. 101—131).

G. PEER, J. JUHÁSZ and J. BÁLINT: "Experimental aseptic necrosis of the bone" (pp. 133—139).

B. FALUDI: "The effect of phenoxyacetic acid on the growth and herbicide resistance of plant tissues" (pp. 141—148).

All papers are well documentized and published in an excellent form.

LÁSZLÓ J. TÖRÖK (Budapest)

"Comparative Neurochemistry", Proceedings of the Fifth International Neurochemical Symposium, St. Wolfgang, Austria, 1962

Edited by DEREK RICHTER

Pergamon Press, Oxford, London, New York, Paris, (1964), Ł 5.

The function of the nervous system (how the brain works) is one of the most interesting problems of the scientific research in our time. It is a consequence of technical progress that the biological problems of kybernetics aroused a rapid interest also in the nervous mechanisms. The unity aspect of structure and function inevitably stress to investigate the chemical mechanisms in the nervous tissue. In the thorough understanding of the nervous mechanisms of higher animals in chemical aspect of structure and function, the comparative study is the best guide in the jungle of accumulated analytical data.

The International Neurochemical Symposia representing adequate reviews in the field of neurochemistry. The symposia held at Oxford (1954), Biochemistry of the Developing Nervous System; Aarhus (1956), Metabolism of Nervous System; Strasbourg (1958), Chemical Pathology of the Nervous System; Varenna (1960), Regional Neurochemistry. In this series the topic of Comparative Neurochemistry was rightly chosen by the Organizing Committee.

The main topics of the symposium are: I. Functional organization in different species; II. Lipides, proteins and ribonucleic acid, III. Amino acids in different species; IV. Energy metabolism and function; V. Neurosecretory mechanisms; VI. Transmitter substances: distribution and metabolism; VII. Comparative neuropharmacology.

First of all the research workers interested in neurochemistry will use this book as an important reference work, but it will be also interesting for workers in biology, zoology, physiology, pharmacology and behavioural sciences.

KÁLMÁN LISSÁK (Pécs)

Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója Műszaki szerkesztő: Farkas Sándor

A kézirat nyomdába érkezett: 1965. I. 21. – Terjedelem: 10,25 (A/5) ív, 49 ábra

65.60177 Akadémiai Nyomda, Budapest – Felelős vezető: Bernát György

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Acta Biol. Hung., **15**(4), 375—382 (1965)

EFFECT OF ACTINOMYCIN D ON THE DEVELOPMENT OF THE COMPENSATORY HYPERFUNCTION OF THE MYOCARDIUM, KIDNEY AND LIVER

F. Z. MEERSON, N. S. KALEBINA, G. A. MALOV, N. T. SIMONYAN and L. K. ROMANOVA

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(Received March 16, 1964)

Synopsis

The factor, selectively inhibiting the DNA-dependent synthesis of RNA, actinomycin D, inhibits the protein and nucleic acid synthesis and the growth of the organs in compensatory hyperfunction of the myocardium, kidney and liver. This enables us to assume that increase of DNA-dependent synthesis of RNA constitutes an initial and indispensable link in activation of the differentiated cells in continuous increase of their physiological function.

Introduction

The relationship between the physiological function and the activity of the genetic apparatus of differentiated cells plays a decisive role in the rise of growth and division of these cells at the time of the increase of the intensity of their function. It has been shown that hyperfunction of the heart in experimental cardiac disease [11, 14, 15], of a single kidney after nephrectomy [8, 20], of the hepatic lobe after partial hepatectomy [4, 6, 16], of the adrenal cortex in prolonged administration of ACTH [2] is regularly followed by an activation of RNA, protein and DNA synthesis in the cells of these organs. This activation increases the number of chromosome sets, the RNA and protein content in the cell and thus either prepares the mitosis or ensures the increase of cellular size and ploidy; it establishes cellular growth and division and is the base of compensatory hypertrophy and regeneration of the organs.

Previous investigations have shown that the factor determining the activity of the genetic apparatus of differentiated cells is not the function of the organ as a whole but the intensity of function of its structures — IFS, which is equivalent to the quantity of function realized by a unit of the mass of the organ [11].

In the initial stage of the process the hyperfunction is brought about by the non-hypertrophied organ, it is made possible solely by the increase of IFS. This increase in turn causes an activation of the genetic apparatus.

Subsequently this hypertrophy results in distribution of the increased function of the organ within the augmented mass of its functioning structures, IFS decreases to a normal or subnormal level, the activation of the cellular genetic apparatus comes to its end and the hypertrophy is completed.

The conception of IFS, as a determinant of activity of the genetic apparatus of the differentiated cell, was the base for the study of the intracellular mechanism by means of which the level of cellular function may exert an influence upon the activity of the genetic apparatus. Available experimental data suggest [7, 13, 19] that there is a correspondence between the degree of intensity of the function of structures and that of the intensity of their wear, i.e. the intensity of cellular protein breakdown. The products of the protein breakdown, "metabolites of wear", while entering into the cytoplasm, may perform a role of factors-effectors activating the genetic apparatus of the cell by elimination of repression of regulatory genes described in detail by JACOB and MONOD [9, 21].

Further study of the problem will elucidate the significance and succession of shifts developing in the genetic apparatus of the differentiating cell in its activation by the increased IFS. According to the current concept of activity of the genetic apparatus, i.e. the apparatus of protein synthesis in the differentiated cells, informational RNA's are synthesized on the structural genes of chromosomal DNA of these cells; afterwards they enter into the cytoplasm, where in the ribosomes, consisting of highly polymerized RNA, they serve as a matrix for the protein synthesis which is brought about with the indispensable aid of S—RNA. Recently obtained data suggest that not only the synthesis of informational RNA but also that of the highly polymerized and S—RNA is a DNA-dependent process [8]. In this connexion it may be assumed that the increase of DNA-dependent synthesis of RNA is an initial and indispensable link of activation of the genetic apparatus of the cell in increase of IFS, a necessary link in the mechanism ensuring growth and division of the differentiated cells in hypertrophy of the organs.

If this conception is valid, then the injection of actinomycin D, selectively inhibiting DNA-dependent RNA synthesis [3, 18, 17], into the animal organism would prevent the activation of RNA, protein and DNA synthesis in compensatory hyperfunction of the heart and inhibit the mitotic activity and the development of hypertrophy of these organs.

In the present work we have investigated the action of actinomycin D* on the protein synthesis and the content of nucleic acids in the myocardium in compensatory hyperfunction of the heart and on the mitotic activity of the hepatic and renal tissues in the hyperfunction of these organs.

* We have used actinomycin D manufactured in the USSR under the name of chyrsomalin. The preparation was kindly presented to us by Professor G. P. MENSHIKOV from the Institute of Experimental Clinical Oncology of the Academy of Medical Sciences.

Material and method

The first series of experiments, in which the effect of actinomycin D on the development of compensatory hyperfunction of the heart has been examined, was performed on 27 albino male rats 175—200 g of weight. The compensatory hyperfunction of the heart (C.H.H.) was produced by constriction of abdominal aorta, below the diaphragm until the aorta diameter had been reduced to one third the original diameter, as described earlier [10] the cross-section of aorta being decreased twice. Such extent of stenosis is not followed by post-operative mortality of animals.

The doses of actinomycin administered in our study have not produced any changes in weight or behaviour of intact rats but in animals with the extent of coarctation referred to they resulted in 27% of cases in the development of cardiac insufficiency and death. The myocardium of this group of rats has not been used in our studies.

Actinomycin was injected intraperitoneally. The first injection was made during the operation of aorta, the second — 48 hours, and the third — 72 hours later.

Each injection contained 17.5 γ of actinomycin per 100 g of body weight 4 hours before the end of the experiment — 20 hours after injection of actinomycin the rats were given intravenously S^{35}-methionine in doses of 5 γ per 100 g of body weight. The animals were killed by decapitation and the relative weight of both ventricles of the heart corrected according to ALEXANDER [1] was indicated. From the homogenate of the myocardium of the left ventricle in every rat the protein was precipitated by the usual method and the incorporation of S^{35} methionine was determined by end-window counter. The concentration of DNA and RNA in the myocardium of the left ventricle was evaluated by the method of SCHMIDT and TANHAUSER modified by A. S. SPIRIN [21]. The nucleic acid content was determined in averaged homogenates of myocardial tissue in each group of rats.

The second series of experiments, in which effect of actinomycin D on the compensatory hyperfunction of the kidney has been studied, was performed on 28 albino male mice 15—20 g of weight. The compensatory hypertrophy of the right kidney was produced by nephrectomy of the left kidney. Actinomycin was injected once, immediately after operation in doses of 25 γ per 100 g of weight; the animals were sacrificed 48 hours after the onset of hyperfunction of the kidney.

The third series of experiments, in which the effect of actinomycin D on the regeneration of the remaining part of the liver after partial hepatectomy was studied, was also performed on 28 albino male mice 15 — 20 g of weight. Hyperfunction of the hepatic portion was produced by removal of two third of it by the method of HIGGINS and ANDERSEN [5]. Immediately after operation actinomycin D was injected in doses of 25 γ per 100 g of weight and 48 hours later once more at the same dosage rate. The animals were sacrificed 72 hours after the onset of hyperfunction of the liver. In animals of I and II series sacrificed after the end of the experiment the relative weight of organs was evaluated, i.e. the relative weight of left ventricles of the heart in rats of the first series and that of the single kidney in mice of the second series. The relative weight was evaluated as the relation of the weight of the organ with blood washed out to the body weight of the animal before sacrifice.

The organs of animals of all three series, were fixed in formol and embedded in paraffin. Sections of 7 μ thickness were stained with hematoxylin-eosin, the number of mitoses per 4,000 cells of the cortical renal layer and in the part of the liver non-adjacent to the wound was counted.

The animals of each of the 3 series were divided into 4 groups: intact animals which were not given actinomycin D (I), experimental animals with compensatory hyperfunction of the organ which were not given actinomycin D (II), intact animals which were given actinomycin D (III) and animals with compensatory hyperfunction which were given actinomycin D (IV). The work is based on the correlation of data obtained in these 4 groups.

Results

The data showing the effect of actinomycin D on the compensatory hypertrophy of the heart are summarized in Table 1.

The Table shows that the hyperfunction of the heart, which was not followed by injection of actinomycin D, resulted in increase of concentration

of RNA in the myocardium by 32.6 per cent, of the intensity of protein synthesis by 43.6 per cent and of relative weight of ventricles of the heart by 21 per cent. The concentration of DNA in the myocardium has not changed and consequently its total content in the myocardium augmented proportionally to the mass of ventricles by 20 per cent. Thus, under the influence of hyperfunction a marked activation of RNA, protein and DNA synthesis and an increase of the mass of the organ were observed. In animals, which at the time of hyperfunction were injected actinomycin D, the mass of the myocardium,

Table 1

Effect of actinomycin D on the protein synthesis and nucleic acid content in the myocardium of the left ventricle 4 days after the onset of CHH

Animal groups / indexes	Control I	Animals with CHH II	Animals with CHH which were given actinomycin D III	Intact animals which were given actinomycin D IV	Authenticity of differences
Number of animals	5	6	9	7	
Relative weight of ventricles of the heart $\left(\frac{\text{Weight of ventricles}}{\text{Body weight}}\right)$	0.00270 $\pm$0.00005	0.00330 $\pm$0.00014	0.00300 $\pm$0.00005	0.00300 $\pm$0.00014	$P_{I-II} < 0.01$ $P_{I-III} > 0.01$ $P_{I-IV} > 0.1$
Incorporation of methionine imp/min/mg	38.5 $\pm$4.5	55.3 $\pm$4.3	37.0 $\pm$3.5	37.0 $\pm$1.9	$P_{I-II} < 0.01$ $P_{I-III} > 0.05$ $P_{I-IV} > 0.05$
DNA in γ per 1 g of tissue	1627	1620	1630	1662	—
RNA in γ per 1 g of tissue	3560	4721	3653	—	—

the content of nucleic acids in the latter and the rate of protein synthesis did not differ from the control.

Thus it follows that the preparation inhibiting the DNA-dependent synthesis of RNA, eliminated the activation of the genetic apparatus of myocardial cells caused by the increase of intensity of function of their structures.

The findings concerning the effect of actinomycin D on the mitotic activity and the weight of the kidney in its hyperfunction are presented in Table 2.

The table shows that 48 hours after the onset of hyperfunction the number of mitoses in the renal tissue increases more than threefold, while the mass of the single kidney augments by 42.2 per cent. Inhibition of DNA-dependent

Table 2

Effect of actinomycin D on the mitotic activity of renal tissue and the weight of kidney 48 hours after the onset of its hyperfunction

Animal groups / Indexes	Intact animals I	Animals with hyperfunction of the kidney II	Animals with hyperfunction of the kidney which were given actinomycin D III	Intact animals which were given actinomycin D IV	Authenticity of distinction
Number of animals	6	6	9	7	
Number of mitoses per one thousand of cells (MK% ± m)	0.25 ± 0.016	1.12 ± 0.052	0.28 ± 0.018	0.65 ± 0.017	$P_{I-II} < 0.1$ $P_{I-III} > 0.1$
Relative weight of the right kidney $\left(\frac{\text{Weight of the kidney}}{\text{Body weight}}\right)$	0.71 ± 0.020	1.01 ± 0.042	0.79 ± 0.036	0.65 ± 0.029	$P_{I-II} < 0.01$ $P_{I-III} > 0.1$

synthesis of RNA by actinomycin fully prevents this increase of mitotic activity and the mass of the organ.

The data presented in Table 3 show that similar results are obtained in the course of study of the effect of actinomycin D on the regeneration of the liver.

Table 3

Effect of actinomycin D on the mitotic activity of the hepatic tissue 72 hours after partial hepatectomy

Animal group / Indexes	Intact animals I	Animals with hyperfunction of part of the liver II	Animals with hyperfunction of a part which were given actinomycin D III	Intact animals which were given actinomycin D IV	Authenticity of distinction
Number of animals	5	7	9	7	—
Number of mitoses per one thousand of cells (MK% ± m)	0.13 ± 0.15	9.7 ± 1.41	0.07 ± 0.42	0	$P_{I-II} < 0.001$

3 days after the onset of hyperfunction of the remaining part of the liver the mitotic activity of the hepatic tissue increased 60—70 times and attained a very high level. Injection of actinomycin D completely inhibited the mitotic activity of the remaining part of the liver.

Discussion

The main result of the experiment consists in the finding that the factor actinomycin D, selectively inhibiting the DNA-dependent synthesis of RNA, fully inhibits the activation of RNA, protein and DNA synthesis in the myocardium in the early stage of compensatory hyperfunction of the heart and prevents the increase of mitotic activity in the kidney and the liver in the early stage of compensatory hypertrophy of these organs. Under conditions of inhibited DNA-dependent synthesis of RNA the increase of IFS does not produce any activity of the genetic apparatus of the differentiated cells.

In evaluating this fact one must bear in mind that under normal conditions the increase of DNA-dependent synthesis of RNA is necessary to ensure both interrelated processes.

First: the DNA-dependent synthesis of RNA is an indispensable precondition for the synthesis of protein ferments non-specific for the given tissue ensuring the formation of desoxyribosid-5-triphosphates and then of new DNA on the matrix of the old one and possibly stimulating the activity of energy — producing structures — mitochondria similarly constituted in different tapes of cells. In the intact cells the synthesis of ferments and initial products necessary for the formation of DNA precedes the initiation of synthesis of DNA itself; it is effectuated in the most protracted period of the interphase of variable duration, known as the phase G_1. It is clear that disturbance of synthesis of the factors necessary for the formation of DNA under the influence of actinomycin D makes impossible the synthesis of DNA itself. This prevents the possibility of transition to mitosis or of increase of the ploidy of the cell in increase of its physiological function.

Secondly: activation of DNA-dependent synthesis of RNA is an indispensable factor ensuring the synthesis of proteins specific for the given cell and playing an immediate role in accomplishement of its physiological function, i.e. of myosin, actin, hemoglobin a.s.o. Ceasing of formation of these and other proteins under conditions of inhibited DNA-dependent synthesis of RNA excludes the activation of protein synthesis in the differentiated cells and makes impossible their growth under the influence of hyperfunction.

It is of essential importance that inhibition of DNA-dependent synthesis of RNA might not prevent the activation of protein and DNA synthesis if there existed considerable reserves of continuously living informational RNA in the differentiated cells of the investigated organs. The fact that inhibition of activation of the protein synthesis and mitotic activity was complete some days after the onset of the hyperfunction of the organs, while by that time the mass of the organs was but inconsiderably augmented, enables us to assume that differentiated cells in mammals do not contain due resources

of informational RNA sufficient for ensuring their growth and division under conditions of hyperfunction.

This requires further study.

Actually we are restricted to concluding that the increase of DNA-dependent synthesis of RNA constitutes an initial and indispensable link in activation of the genetic apparatus of the differentiated cells in continuous increase of their physiological function.

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DIE ROLLE DES AKTINOMYZIN-D BEI DER KOMPENSATORISCHEN HYPERFUNKTION VON MYOCARD, NIERE UND LEBER

Aktinomyzin D — der die DNS-abhängige RNS-Synthese selektiv blockierende Faktor — hemmt die Eiweißsynthese, die Synthese der Nukleinsäuren und das Organwachstum, bei kompensatorischer Hyperfunktion des Myokards, der Leber und der Nieren. Diese Tatsache legt die Vermutung nahe, daß die Erhöhung der DNS-abhängigen RNS-Synthese ein primäres und notwendiges Glied in der Aktivierung des genetischen Apparates bei der anhaltenden Steigerung der physiologischen Funktion von differenzierten Zellen ist.

РОЛЬ АКТИНОМИЦИН Д В РАЗВИТИИ КОМПЕНСАТОРНОЙ ФУНКЦИИ МИОКАРДА, ПОЧЕК И ПЕЧЕНИ

Фактор, избирательно блокирующий ДНК-зависимый синтез РНК — актиномицин Д — тормозит синтез белка, нуклеиновых кислот и рост органов, при компенсаторной гиперфункции миокарда, почек и печени. Это дает основание полагать, что увеличение ДНК-зависимого синтеза РНК составляет первоначальное и необходимое звено активации генетического аппарата дифференцированных клеток при длительном увеличении их физиологической функции.

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Acta Biol., Hung., **15**(4), 383—392 (1965)

EXPERIMENTAL INVESTIGATIONS ON NEUROSECRETION IN MUDFISH (MISGURNUS FOSSILIS L.)

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(Received July 5, 1964)

Synopsis

The authors studied experimentally the neurosecretory activity of the praeoptic nucleus in the mudfish, *Misgurnus fossilis*, kept in media of different osmotic pressure (lake-water, 2% NaCl, distilled water, out of water, etc). The changes in activity were studied by histological methods. The two parts of the praeoptic nucleus, i.e. the magno- and parvocellular parts behaved differently. Hypotonic medium gave rise to an increase in the quantity of neurosecretory substance in the magnocellular part and loss in the same substance in the parvocellular part of the praeoptic nucleus. Dehydration resulted in loss in the neurosecretory substance in the parvocellular part.

Introduction

In the diencephalon of the Teleosts there are two neurosecretory hypothalamo-hypophyseal systems, generally well developed, in all species studied till now: the system of the praeoptic nucleus and that of the lateral nucleus of the tuber. The morpho-functional connexion of these nuclei is accomplished by the hypothalamo-hypophyseal tract.

The morphological phenomena of the neurosecretory activity of these two systems have been studied in detail. The functional significance of these systems, however, has not been elucidated satisfactorily. On the basis of observations in other vertebrates it is believed that these neurosecretory systems are of importance in the water and electrolyte metabolism. In respective experiments [2, 7, 12, 15] various species of fresh-water, brack-water and marine fishes, were kept in hyper- or hypotonic solutions for various periods of time and subsequently, the neurosecretory activity of the respective nerve cells was studied. The majority of the authors found a remarkable loss in neurosecrete in fishes kept under hypertonic conditions, but others could not confirm this observation [15]. Under hypotonic conditions cumulation of the neurosecrete was observed in certain species, whereas in others its amount decreased. In general, a great individual variability was observed both under physiological and experimental conditions.

In the course of investigations on the hypothalamic systems of neurosecretion in *Cyprinidae* we found a seasonal variation of neurosecretory activity of the praeoptic nucleus [14] and considerable individual variations within the species studied. Obviously the neurosecretion by the diencephalon of Teleosts has not been elucidated sufficiently.

In this paper we report on our experiments concerning the neurosecretory activity of the praeoptic nucleus of the mudfish (*Misgurnus fossilis* L.). The mudfish was chosen for the following reasons: (1) In this species the neurosecretory system has not been studied in detail; (2) "intestinal respiration" makes mudfish able to live in waters of low oxygen concentration; (3) we supposed that this species easily tolerates large osmotic variations.

We wished to study the modifications in the neurosecretory activity of the nerve cells of the praeoptic nucleus under the influence of the medium.

Material and method

Adult specimens of mudfish (*Misgurnus fossilis* L.) were fished from the Transsylvanian Plain lakes in November 1963. The specimens were nearly uniform in length and weight, i.e. 23 cm and 65—75 g, respectively. Thirty fishes were divided in six equal groups. The individuals of group I were killed immediately after having caught. Group II was kept in 2% NaCl solution for two hours. Group III was kept in tap-water for two hours; group IV in distilled water for 48 hours; group V was kept out of water at room temperature for two hours, group VI in tap-water at 32°C for 72 hours (Table 1).

The fishes kept in 2% NaCl hardly tolerated this high concentration and after two hours were hardly able to move. Those kept out of water did not show any sign of asphyxia. The fishes kept in tap-water and those kept in distilled water tolerated the experimental conditions well and those kept in the incubator at 32°C or in tap-water moved more vividly than normal ones.

At the end of the experiment all fishes were sacrified. The brains with the buccal part of the skull were fixed in Bouin's fluid. After fixing they were taken from the remnants of the skull and embedded in paraffin. Sections 5—7μ in thickness were stained by Gömöri's chrome-haematoxylin phloxine.

Results

The praeoptic nucleus of mudfish (*Misgurnus fossilis* L.) like that of all species of Teleosts is composed of two distinct parts, *viz.* a dorsal part called *pars magnocellularis* (PMC) and a ventral part, *pars parvocellularis* (PPC). The position and proportionality of these are, in general, nearly the same as in other Teleosts. The only difference is that the cells of the PPC are situated immediately under the ependyma whereas between the ependyma and PMC there is a layer consisting of nervous fibers, glial elements and dilated capillaries.

The nerve cells of the PMC are spread losely showing large gaps with dilated capillaries. There are only few cells (4—5) in the dorsal region of the PMC; their number gradually arises towards the ventral part. There are differ-

ences in size of cells between the dorsal and the ventral parts of the PMC. The formers range from $10 \times 25\ \mu$ to $25 \times 30\ \mu$, the latters from $12 \times 15\ \mu$ to $15 \times 18\ \mu$.

The cells of the PPC are densely packed giving little space for nerve fibres and blood capillaries.

In the following we describe the histologic and cytologic characteristics observed in the neurosecretory activity of the praeoptic nucleus in the individuals of the six groups.

(1) In individuals caught from *lake-water*, i.e. in the *normal animals*, in the praeoptic nucleus only few cells contain neurosecretory granules in the cytoplasm. Most of the cells are deprived of Gömöri-positive granules (Fig. 1.). The nerve cells which contain granules have a spheric nucleus, $5-7\ \mu$ in diameter, in central position. The nucleolus is well-shaped, weakly basophilic, and the nuclear chromatin is relatively abundant. The cytoplasm surrounding the nucleus in a thin layer is intensely stained with chrome-haematoxylin. In the intercellular spaces there are numerous dilated blood-capillaries surrounded by accumulated neurosecretory substance.

Nuclear polymorphism characteristic of the majority of the Teleosts examined cannot be recognized in the PMC.

The cells of the PPC are close to one other. Each has a spherical, relatively large nucleus, surrounded by a thin layer of cytoplasm.

The number of cells showing neurosecretory granules is higher in the PPC than in the PMC. In these cells the perinuclear zone of cytoplasm contains numerous neurosecretory granules. In the intercellular spaces, besides of dilated capillaries, nervous fibers and glial elements are present.

(2) The mudfishes kept in 2% *solution of NaCl* hardly tolerated this salt concentration. After 1.5 hours from the beginning of the experiment the mudfishes moved weakly and by two hours one of them had died. The four survivors were sacrificed.

For the general stasis in the cerebral vessels and capillaries there are also in the praeoptic nucleus a great number of dilated capillaries full of blood.

The nerve cells of the PMC are smaller than in normal animals, their maximum being $10 \times 15\ \mu$. In most cells the cytoplasm shows no neurosecretory granules. Only very few cells contain negligible amounts of granules in the perinuclear zone (Fig. 2). The granules are large and very intensely stained.

The histological picture of the PPC of the praeoptic nucleus highly differs from that observed in PMC. The great quantity of Gömöri-positive cytoplasmic granules (Fig. 3) make these cells large (cca. $15 \times 20\ \mu$) with a large spherical nucleus each. In the nucleus there are very few chromatin granules. Besides these large cells there are small ones ($8-10\ \mu$ in diameter) which contain, but a small quantity of neurosecretory granules in the perinuclear zone. We did not observe extracellular neurosecretory granules.

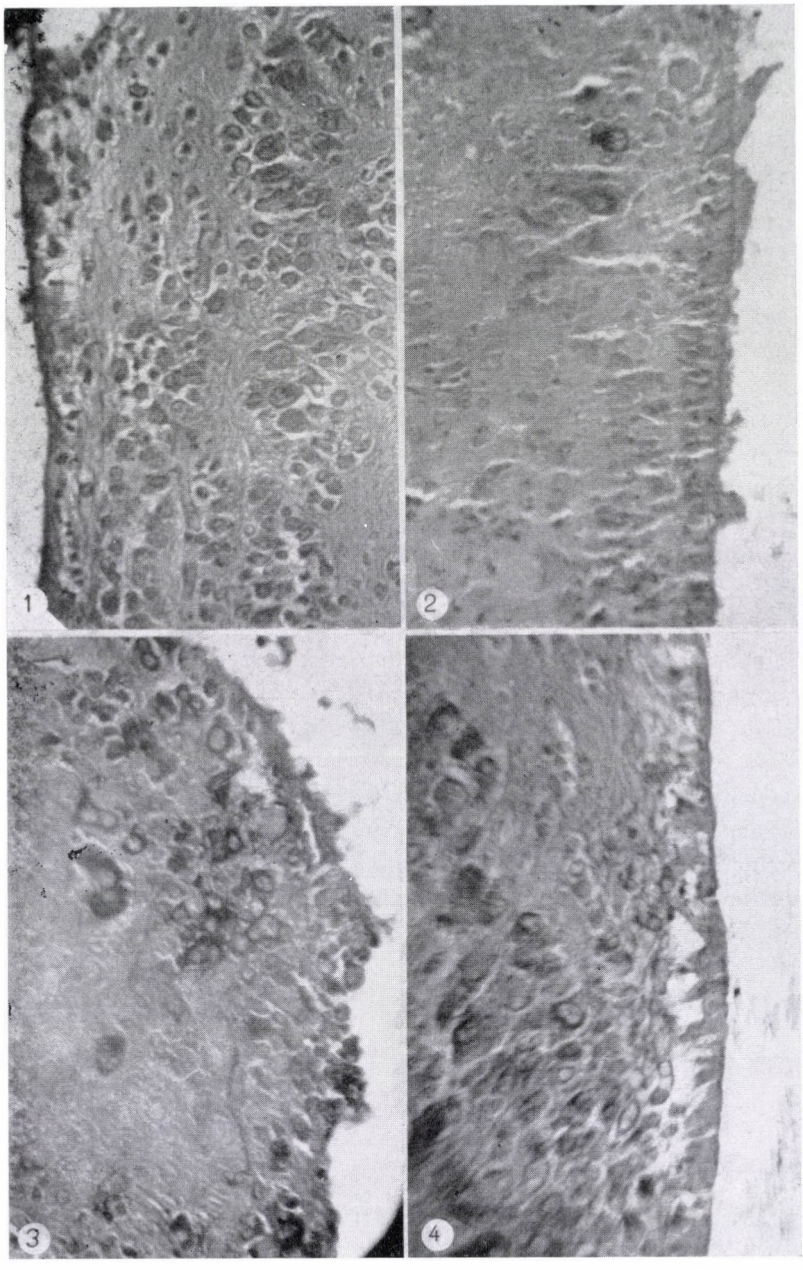

Fig. 1. Pars magnocellularis nuclei praeoptici in a normal mudfish. Transverse section. The nerve cells contain few neurosecretory granules Gömöri's chrome-haematoxylin-phloxine stain, × 25

Fig. 2. Pars magnocellularis nuclei praeoptici in *Misgurnus fossilis* kept in 2% NaCl for two hours. Transverse section. Neurosecrete in the cells is decreased in amount. Only some cells contain neurosecretory granules. Gömöri's chrome-haematoxylin-phloxine stain, × 25

(3) The *control animals* were kept in tap-water for two hours at room-temperature. The histological aspects of the two parts of the praeoptic nucleus are very different in these fishes.

The majority of the cells in the PMC contain numerous neurosecretory granules not only in the perinuclear zone but also in the peripheral regions of the cytoplasm. The nuclei are swollen, 10—14 μ in diameter, they are perfectly spherical. Dilated capillaries are seen, especially in the dorsal region of this part.

The small cells of the PPC contain no neurosecretory granules. These are shown by only a very reduced number (about 5%) of nerve cells, the larger ones. These cells are disseminated among the small ones and have a relatively large, optically empty nucleus. Some of them have granules only in the perinuclear zone of the cytoplasm; in others the whole cytoplasm is full of neurosecretory granules. Nuclear polymorphism is observable neither in the PMC nor in the PPC.

(4) The animals kept for 48 hours in *distilled water* tolerated this medium very easily. The cells in the PMC are smaller than those in normal individuals. Many cells contain neurosecretory granules and stain intensely in comparison with cells deprived of granules. The nucleus in the cells containing granules are swollen, 8—10 μ in diameter. The granules accumulate mainly in the perinuclear zone. The blood capillaries are greatly dilated.

In the PPC most cells are deprived of granules. In these cells the relatively large nucleus is surrounded by a thin layer of cytoplasm. In this part there are some cells containing cytoplasmic neurosecretory granules. The nucleus of these cells is large and optically nearly empty. It is surrounded by a cytoplasm very rich in neurosecretory granules. These are spread uniformly in the whole cytoplasm. We observed no extracellular neurosecretory granules.

(5) The mudfishes kept *out of water* moved vividly even after two hours and did not show any sign of asphyxia. They secreted a great quantity of mucus, which formed a protecting layer around them.

In these individuals the two parts of the praeoptic nucleus obviously contain different quantities of neurosecretory substance. The dimension of the neurosecretory cells in the PMC vary from 12×15 to 15×25 μ i.e. in the same range as in the normal ones. The nuclei of 8—10 μ in diameter have central or eccentric position. The number of the nerve cells containing cytoplasmic granules is much higher, than in normal individuals. (Fig. 4)

Fig. 3. Pars parvocellularis nuclei praeoptici in *Misgurnus fossilis* kept in 2% NaCl. Transverse section. The majority of cells is full of Gömöri-positive granules Gömöri's chrome-haematoxylin-phloxine stain, × 25

Fig. 4. Pars magnocellularis nuclei praeoptici in *Misgurnus fossilis* kept out of water. Accumulation of neurosecretory granules in some cells. Gömöri's chrome haematoxylin-phloxine stain, × 25

Especially the large nerve cells of the dorsal regions of the PMC show an accumulation of great amounts of cytoplasmic neurosecretory granulation. The nucleus has an eccentric position and the neurosecretory granules fill the whole cytoplasm. In the ventral regions of the PMC, the cells are smaller and contain fewer neurosecretory granules. The capillaries are moderately dilated.

In the PPC the majority of the nerve cells contain no Gömöri-positive substance, but there are also some highly-granulated hypertrophic cells. The

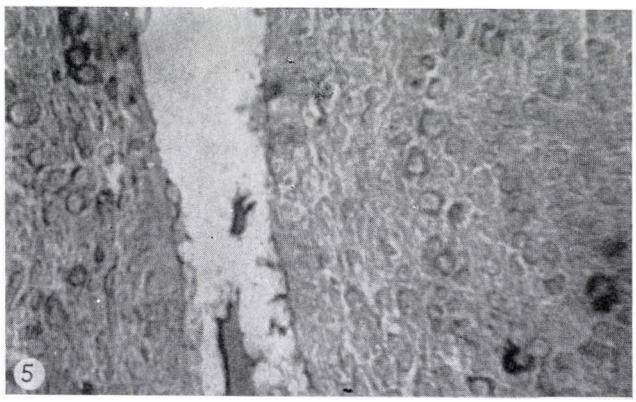

Fig. 5. Pars magnocellularis nuclei praeoptici in *Misgurnus fossilis* kept at 32°C. Neurosecretory granules only in a few cells. Gömöri's chrome haematoxylin-phloxine stain, × 25

coarse granules of these cells being dispersed in the whole cytoplasm stain very intensely.

Around the dilated capillaries accumulation of neurosecretory substance is often seen. Between the granulated and ungranulated cells the difference is very sharp.

(6) The individuals of mudfish kept in *tap-water at 32°C for 72 hours* tolerated this elevated temperature very easily and the low oxygen content of water did not provoke any sign of respiratory perturbation.

The neurosecretory substance is very reduced in both parts of the praeoptic nucleus. The great majority of the PMC shows no neurosecretory granules. In the few granulated cells the granules (Fig. 5) take place in a thin layer of the perinuclear zone. Around the extremely dilated capillaries there is often a layer of Gömöri-positive substance.

The nerve cells of the PPC are also deprived of granules except a few large (18—22 μ in diameter) cells (5—6 altogether), the cytoplasm of which is full of neurosecrete. In this regard the cytologic aspect of the PPC is very similar to that observed in animals of Group V.

Discussion

The neurosecretory hypothalamo-hypophyseal system of the mudfish (*Misgurnus fossilis* L.) had not been studied either from morphological or from histological point of view. SCHIEBLER and HARTMANN [12], who studied four specimens of mudfish, found no essential morphological difference in the neurosecretory hypothalamo-hypophyseal system between mudfish and other Teleost species.

The two parts of the praeoptic nucleus (the magnocellular and parvocellular parts) are clearly distinguishable from each other. The nerve cells of the PMC are smaller than the respective cells of other Teleost species. Another characteristic of this part is that the cells are spread showing wide spaces between one other.

The cells of the PPC of the praeoptic nucleus are extremely small (8—12 μ in diameter). In these cells the relatively large nucleus is surrounded by a thin layer of cytoplasm. In the dorsal region of the PPC there are some larger cells as reported also by SCHIEBLER and HARTMANN [12].

The quantity of the neurosecretory substance in these two parts of the praeoptic nucleus is scarce showing that the mudfish belongs to the Teleosts being poor in neurosecrete.

Nuclear polymorphism, a phenomenon characteristic of the neurosecretory cells of the praeoptic nucleus of Teleosts, was observed neither in the PMC, nor in the PPC. In this regard the praeoptic nucleus of the mudfish is like that observed in the Hungarian barbe (*Barbus meridionalis* petényi HECK) [14].

In the normal (untreated) animals the number of cells with neurosecretory substance is higher in the PPC than in the PMC.

In the last years some authors [5, 6, 11] described that the praeoptic nucleus of *Amphibians* is composed of several distinct functional regions. According to these authors the praeoptic nucleus of the *Batrachia* is composed of a dorsal region and two ventral regions each having a different significance in the osmoregulation.

The results of our investigations prove conclusively that the two parts of the praeoptic nucleus (PMC and PPC) give different response to osmotic stimuli. The neurosecretory cells of the PMC in individuals kept in a hypertonic medium (2% NaCl) show no appreciable loss of neurosecretory granules in contrast to the respective cells in nontreated individuals, while in the PPC a well-defined retention of the neurosecretory substance is recognizable.

Our findings agree with those of TUURALA [15], who kept *Gasterosteus aculeatus* specimens in 2% NaCl solution and could not demonstrate any significant change in the neurosecrete content of the praeoptic nucleus. Other investigators, on the other hand, have published that keeping fresh-water fishes in 2% NaCl solution for 1—2 hours provoked a considerable release of the neuro-

secretory substance from the praeoptic nucleus [1]. In our opinion the latter phenomenon might be considered as a stress effect on the organism, provoked by a nonphysiological, harmful environmental alteration.

Since the mudfishes survived only for a short time, at most two hours, in 2% NaCl solution the little difference from the normal state cannot be considered as evidence of the significance of osmoregulation by the neurosecretory cells of the praeoptic nucleus.

The morphological changes in the praeoptico-hypophyseal system of mudfishes kept for a longer time in tap-water or in distilled water are more convincing. In all individuals of both groups some PMC cells contained an excess of neurosecretory granules and as compared to the controls significantly more PMC cells contained neurosecretory granules at all than in the control individuals.

Thus, a retention of the neurosecretory substance is evident in these cases. Our observations are in good accord to those of ARVY and GABE [2] in *Callionymus lyra* and *Ammodytes lanceolatus*, and with those of TUURALA [15] in *Phoxinus laevis* and *Gasterosteus aculeatus* on the accumulation of neurosecretory substance under the influence of hypotonic media.

The present data disprove the general validity of the findings of KORN [8] and SCHIEBLER and HARTMANN [12]. These authors found reduced neurosecretion in the praeoptic nucleus in *Mugil*, *Gobius* and *Anguilla* having been kept in hypotonic medium.

It should be noted that the nerve cells of the PPC in the animals of groups III, IV and V contained very minute amounts of neurosecretory granules. The cytoplasm of these cells was generally deprived of Gömöri-positive granules except a few (2 or 3) larger cells that contained neurosecretory granules. Thus keeping in hypotonic medium led to the disappearance of neurosecrete from the PPC cells.

In mudfishes kept at 32°C for a longer time only little amounts of neurosecretory substance were found both in the magnocellular and in the parvocellular parts of the praeoptic nucleus. Only some larger cells of the dorsal region of the PPC showed neurosecretory granules. In this case therefore there exists a difference in the quantity of neurosecrete between the two parts of the praeoptic nucleus.

In every group there was a considerable difference in the amount of neurosecretory substance between the two parts of the praeoptic nucleus. When PMC was rich in this substance, PPC was almost free of it and *vice versa*. It can thus be concluded that the fluctuation of the neurosecretory substance in the two parts of the praeoptic nucleus is asynchronous or even opposite.

The results of the present investigations are not conclusive concerning the significance of the neurosecretory activity of the praeoptic nucleus in

osmoregulation. We can state that in hypotonic medium, or as a result of dehydration of the organism, a retention or accumulation of the neurosecretory substance takes place in the PMC of the praeoptic nucleus.

The divergent morpho-physiological manifestations of the two parts might be interpreted differently:

(1) the two parts may have different functions;

(2) the two parts may have the same physiological function but their function is asynchronous;

(3) the release of the granules takes place first in the dorsal part of the praeoptic nucleus and gradually spreads to the ventral part;

(4) the retention of neurosecretory substance may start in the magnocellular part and the parvocellular part may be involved only in a later stage.

To establish the validity of one of these suppositions further experiments are necessary.

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EXPERIMENTELLE UNTERSUCHUNGEN ÜBER DIE NEUROSEKRETION BEI *MISGURNUS FOSSILIS* L.

Es wurde die Neurosekretion des Nucleus praeopticus von *Misgurnus fossilis* L. experimentell untersucht. Die Versuchstiere wurden verschiedenen osmotischen Verhältnissen der Umwelt ausgesetzt (Seewasser, 2%ige NaCl-Lösung, Aqua dest., Haltung am Trockenen usw.). Die Änderungen der Neurosekretion wurden histologisch untersucht. Es konnte festgestellt werden, daß die beiden Teile des Nucleus praeopticus die Pars magnocellularis und die Pars parvocellularis Änderungen der osmotischen Verhältnisse der Umwelt verschieden beantworten. In hypotonischer Umwelt nimmt die Menge des Neurosekretes in der Pars magnocellularis zu. In hypertonischer Umwelt bzw. am Trockenen nimmt sie in der Pars parvocellularis ab.

ЭКСПЕРИМЕНТАЛЬНОЕ ИССЛЕДОВАНИЕ НЕВРОСЕКРЕЦИИ *MISGURNUS FOSSILIS* L.

Авторы изучали невросекреторную деятельность преоптического ядра *Misgurnus fossilis* L. при помощи экспериментального метода.

Подопытные животные держались в различных осмотических условиях (озерская вода, раствор хлористого натрия 2%, дистиллированная вода, сухая среда). Изменения невросекреторной деятельности исследовали гистологическим методом. Установлено, что два части преоптического ядра именно крупноклеточная и мелкоклеточная часть реагируют в своей невросекреторной деятельности на осмотические изменения среды разным образом. Под влиянием гипотонической среды количество невросекрета увеличивается в крупноклеточной части. Но в гипертонической или сухой средах количество невросекрета в мелкоклеточной части преоптического ядра понижается.

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Acta Biol. Hung., **15**(4), 393—408 (1965)

GLOMERULOMETRICS II

NEW DATA CONCERNING THE GROWTH OF GLOMERULI IN THE ALBINO RAT

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(Received: August 27, 1964)

Synopsis

Size and percentage distribution of the renal glomeruli in the cortex have been examined in 85 albino rats of different age groups and of both sexes from birth every week during a span of 15 weeks, with each group consisting of 5 animals. The differences in size between the glomeruli of the cortical zone and those of the juxta-medullary zone have also been studied. The growth of the glomeruli is uneven. After birth a very large number of the glomeruli, much smaller in size than the average of this age, can be seen, which, however, equal in size with the others at the end of the second week. The rate of growth is slow until the beginning of the second week, then there is a period of intensive growth from the 2nd to the 7th week, to slow down again by the following 3 weeks. No essential change in glomerular size can be observed after the loth week. The glomeruli of the juxta-medullary zone are always larger than those of the cortical zone. The difference between them is widest immediately after birth (70%), the loth to the 15th week. The percentage distribution of the glomeruli in the cortex diminishes to a value of 3.7% by the 15th week from the maximum level of 10.1% after birth.

Introduction

Several data referring to the size and number of the glomeruli are available in literature [1, 4, 6, 11]. It is generally accepted that the multiplication of the glomerular surface by the number of glomeruli is proportional to the body surface. This proportion, however, can be different, depending on species [10] and on their habitat [11]. Usually the growth of the glomeruli is in direct ratio to that of the body, but this relation is only valid in general [6].

The size of the glomeruli within one species changes primarily in accordance with age. In rats small glomeruli are found at birth and their distribution in the cortex is dense. They begin to grow rapidly after a period of stagnation during sucking. From the 50th to the 70th day of life the rate of development slows down [1]. In the meantime the density of the glomerular distribution in the cortex decreases. According to ARATAKI [1] the percentage distribution of the glomeruli in the cortex at birth amounts to 12.6% (11% ♀) then by the fiftieth day it goes down to 3% (3.3% ♀) and later it increases slightly again.

KITTELSON [4] states that the percentage distribution of the glomeruli in the cortex of a new-born rat is 13%, and by the 12th week it decreases to 6%.

Some deviation in the curve of the glomerular growth may occur during life in certain species — as in the rat — owing to the development of new glomeruli after birth. This leads to a decrease in the average size [1, 4 and 9].

Views in the literature differ to a considerable extent on the question whether there is a measurable difference, if any, between the glomeruli of the cortex according to their location. It has been generally accepted since BOWMAN's investigation that the periferal glomeruli are smaller then those of the juxta-medullary zone. Although there are also data suggesting that in certain species the periferal glomeruli are of a major size [8]. MORISON [5] distinguishes four types of glomeruli in the kidney of rat according to their position and the area supplied by their efferent vessels. There are: the subscapular, the cortical proper, the corticomedullary and the medullary glomeruli. HEGGIE [3] termed the deep-lying glomeruli as juxta-medullary ones. This term has also been used by TRUETA and co-workers [12], who called the attention to the morphological and functional importance of these glomeruli. The main difference between the juxta-medullary glomeruli and the other cortical glomeruli is, that the efferent arterioles of the former divide into the recta vessels for the medulla, while the latter supply the cortex. The cortical glomeruli correspond to MORISON's [5] first two classes, whereas the juxta-medullary ones to the second two groups. The differences in the size of the juxta-medullary and cortical glomeruli vary according to the species concerned and they change in accordance with age [12].

There is also a difference in the size of the glomeruli between males and females — referred to identical body weight. The glomeruli of the males are larger in size [1].

The dimensions of the glomeruli are determined by the following factors (listed in the order of importance): species, age, position and sex.

Material and method

A total number of 85 albino rats of both sexes were used. The animals were killed for investigation in groups at various intervals, i.e. immediately after birth, at 2, 7 days of age and then at 1 week intervals until 15 weeks of age. In each group 5 rats were taken (Table 1). The animals were killed with ether, then they were weighed. The kidneys were removed and also weighed. The volumetric analysis of the glomeruli and their percentage distribution in the cortex were made according to the methods of PALKOVITS and ZOLNAI [7].

The kidneys were fixed in 4% formalin, embedded in paraffin wax, cut at 17 μ and stained with haematoxylin and eosin. The sections were projected against an opaque glass plate so that the field of view obtained by projection ranged up a magnification of 250 times. Most of the glomeruli appeared in elliptical outlines, therefore in order to obtain their volumes there were taken elliptical bodies rotated along their long axis. The long and short diameters were measured with a millimeter ruler and the volume of the ellipsoides were computed. 300 measurements were made in each kidney. The logarithm of the glomerular volume was taken in order to allow classification. The classified glomerular volumes were demonstrated in a

distribution curve, the logarithmic classes were plotted against the abscisse and the percentage of the glomeruli occurred within the classes, against the ordinate.

Further, on the sections the cortical and the juxta-medullary zones were separated by a line of China ink drown on the cover glass under a stereo microscope. 300 glomeruli were measured in each zone at every kidney in order to determine the difference in size between glomeruli of the two above zones.

The percentage distribution of the glomeruli in the cortex was calculated according to the method of PALKOVITS and ZOLNAI [7]. Sections made of each kidney were projected onto an opaque glass plate at a magnification of 250 times. On the opaque glass plate there was a grid of ten horizontal and ten vertical lines. The number of glomeruli falling within the intersections of the lines was counted. Twenty different fields were counted in each kidney. The average of these numbers obtained was regarded as a ration between glomerular volume and total tissue volume in the renal cortex.

Results

The values obtained in all animals examined are shown in Table 1.

In *newborn* animals the volumetric analysis of the glomeruli shows two striking characteristics. The first one is that the glomeruli of the juxta-medul-

Table 1

Age 0=newborn 3=days 1–15= weeks	Average of glomerular volume measured in the entire cortex, in μ^3	Average of glomerular volume measured in the cortical zone, in μ^3	Average of glomerular volume measured in the juxta-medullary zone, in μ^3	Juxta-medullary-cortical ratio %	Percentage distribution of glomeruli in the cortex
0	4 066	3 132	5 355	71,9	10.11
3	4 272	3 065	5 071	68,7	7.19
1	4 914	4 094	5 623	38,2	8.03
2	5 082	4 103	6 374	57.0	6.82
3	6 371	5 263	7 557	46.1	6.14
4	8 788	6 964	10 077	47.1	4.35
5	10 042	8 313	11 150	35.3	4.50
6	11 602	10 133	12 282	22.0	4.51
7	14 484	12 775	15 964	26.4	4.28
8	14 745	12 656	16 800	28.9	3.87
9	16 800	14 307	19 059	34.3	4.44
10	14 615	13 600	16 157	19.3	4.12
11	17 274	15 376	18 654	23.8	3.76
12	16 409	14 983	18 550	25.5	3.89
13	18 850	17 133	22 067	29.6	3.99
14	17 785	16 583	19 420	16.4	3.75
15	17 209	16 424	18 453	31.1	3.77

lary zone are much larger than those located near the surface. The second one is that the cortical glomeruli can also be classified into two groups according to their small and large sizes. On the distribution curve obtained, two peaks

are seen (Fig. 1/a), which indicate this considerable difference in size. The average volume of the glomeruli computed from the glomeruli of the small type varies between 1,200—1,600 μ^3 as against 3,000—4,000 μ^3 of the large

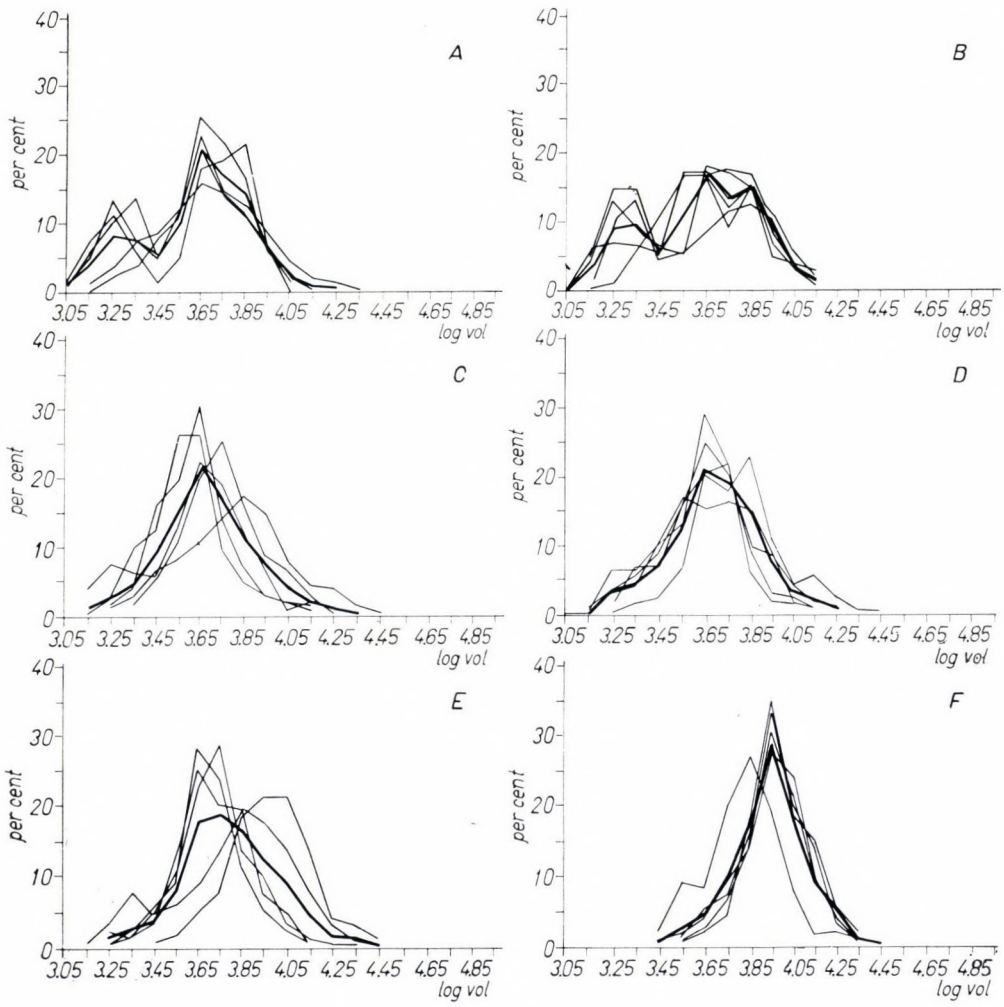

Fig. 1. Distribution curves of the average volume of renal glomeruli

A = newborn C = 1 week old E = 3 weeks old
B = 3 days old D = 2 weeks old F = 4 weeks old

The logarithms of the volume categories is presented horizontally; the number of glomeruli belonging to one category and expressed in percentage is given in the vertical line. The thick curve indicates the average of the glomeruli of animals of the same age

one. This value of the juxta-medullary glomeruli ranges up to 6,000 μ^3. The majority of the glomeruli of a small size can be found in the periphery of the cortex, although some of them can be found in the juxta-medullary zone as

indicated by a peak on the distribution curve of the glomerular volumes at the juxta-medullary zone on the left hand side (Fig. 4/a). The difference in size between the cortical and juxta-medullary glomeruli is 71.9%. This value is the highest among the animals of any age examined. It is remarkable that in the juxta-medullary area glomeruli with a size similar to those of an adult animal can be found even at birth. It is also interesting that the glomeruli of this area appear on sections in large number, their percentage distribution in the cortex is 10.1%. This value is almost three times higher than of the adult animals. The cortex area close to the surface is particularly densely populated by glomerules.

In the group of the 3 *days old* rats, although the growths of the glomeruli is slight (no more than 5.1%), a considerable change can be observed in the area of the small glomeruli close to the surface. They show a most intensive growth after birth, and the peak corresponding to them in the curve shifts to the right with one class — by about 30% — while the rest of the cortical or the juxta-medullary glomeruli are represented in the same groups as they are seen in the new-born animals (Fig. 1/b). It is interesting to note that small glomeruli of the juxta-medullary zone still occur; that is why their distribution curve shows three peaks (Fig. 4/b). The curve is flat and the volume of the glomeruli ranges between 1,000 and 12,000 μ^3. The difference between the average volume of the cortical and juxta-medullary glomeruli is 68.7%. This is practically identical with that of the new-born animals. This stage of the kidney's development is dominated by the growth of the tubules, since the percentage distribution of the glomeruli in the cortex dropped from 10.1% to 7.2%.

In the group of the *one week old* animals, the growth of the glomeruli, the levelling up of differences between the glomerular sizes and the comparatively faster growth of the rest of the cortex, continues, although the rate of development is rather slow at this stage. The volume of the glomeruli has grown by 15%, which is chiefly due to the fact that the small type of glomeruli have become larger, therefore instead of the left peak of the curve, only a small plateau is to be found (Fig. 1/c). The small glomeruli have disappeared from the juxta-medullary zone as indicated by Figure 4/c. The difference in size between the glomeruli of the two zones, is 38.2%, it is much less than in the previous groups.

In the group of the *two weeks old* animals the glomeruli of small size which were seen at birth have completely disappeared; the glomeruli of the juxta-medullary zone are uniform in size. The curve obtained are normal Gauss-curves (Fig. 4/d). The difference in size between the glomeruli of the two zones is 57%, but a considerable fluctuation of values which range from 22.1 to 99.1% can be observed.

In the group of the *three weeks old* animals a pronounced growth of the glomeruli is detectable. The growth during the span of seven days is over 25%.

In both the cortical and juxta-medullary zones glomeruli with a size larger than the average can be observed and a plateau is formed on the right hand side of the curves as indicated by Figure 1/e. The two curves run parallel, the

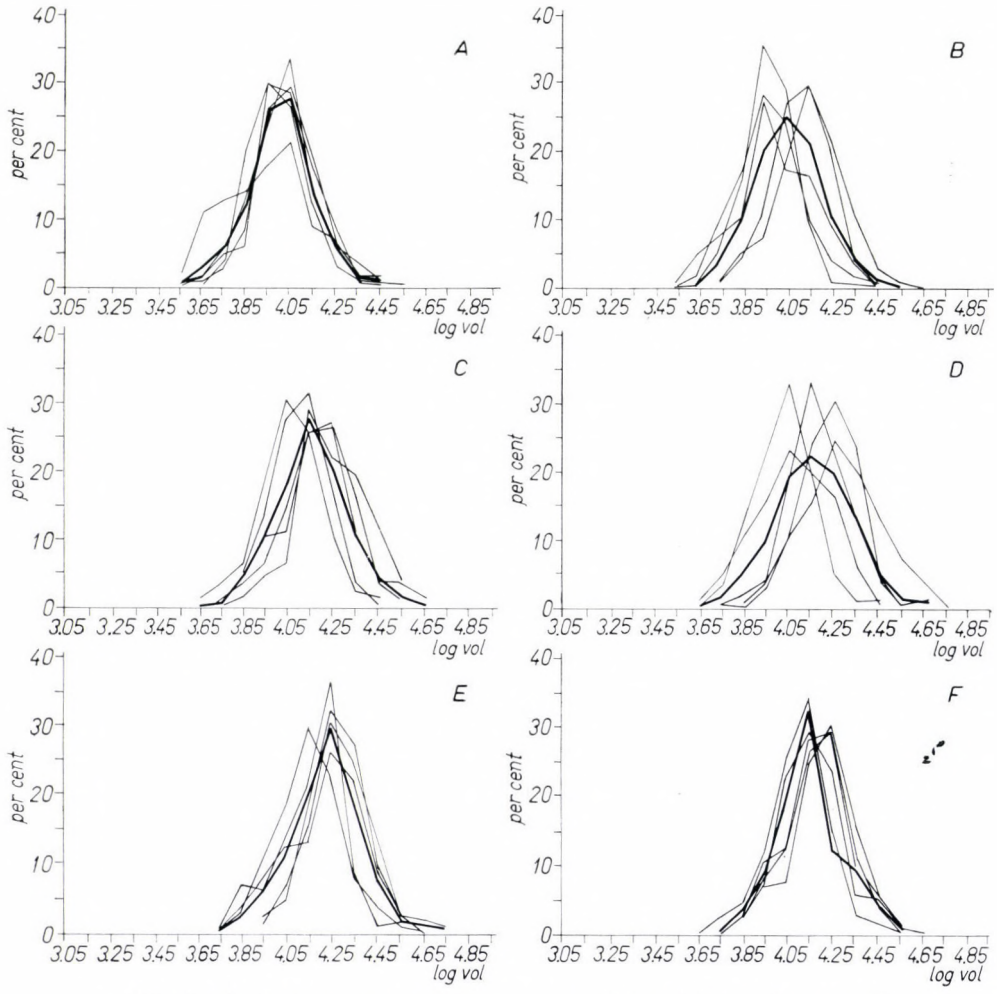

Fig. 2. Distribution curves of the average volume of the renal glomeruli

A = 5 weeks old C = 7 weeks old E = 9 weeks old
B = 6 weeks old D = 8 weeks old F = 10 weeks old

For labelling see Fig. 1

only difference between them is that the juxta-medullary one is shifted to the right with one class, which accounts for a difference of 46% in size (Fig. 4/e). Although growth of the glomeruli is of a very high level, the tubular system develops to an even higher rate, because the percentage distribution of the glomeruli in the cortex stays at 6.14% only.

One of the five animals examined in this group has shown retained general development. Therefore the distribution curve of the glomeruli is of similar type as it has been found in the previous groups. The curve is shifted to the left and there are two peaks.

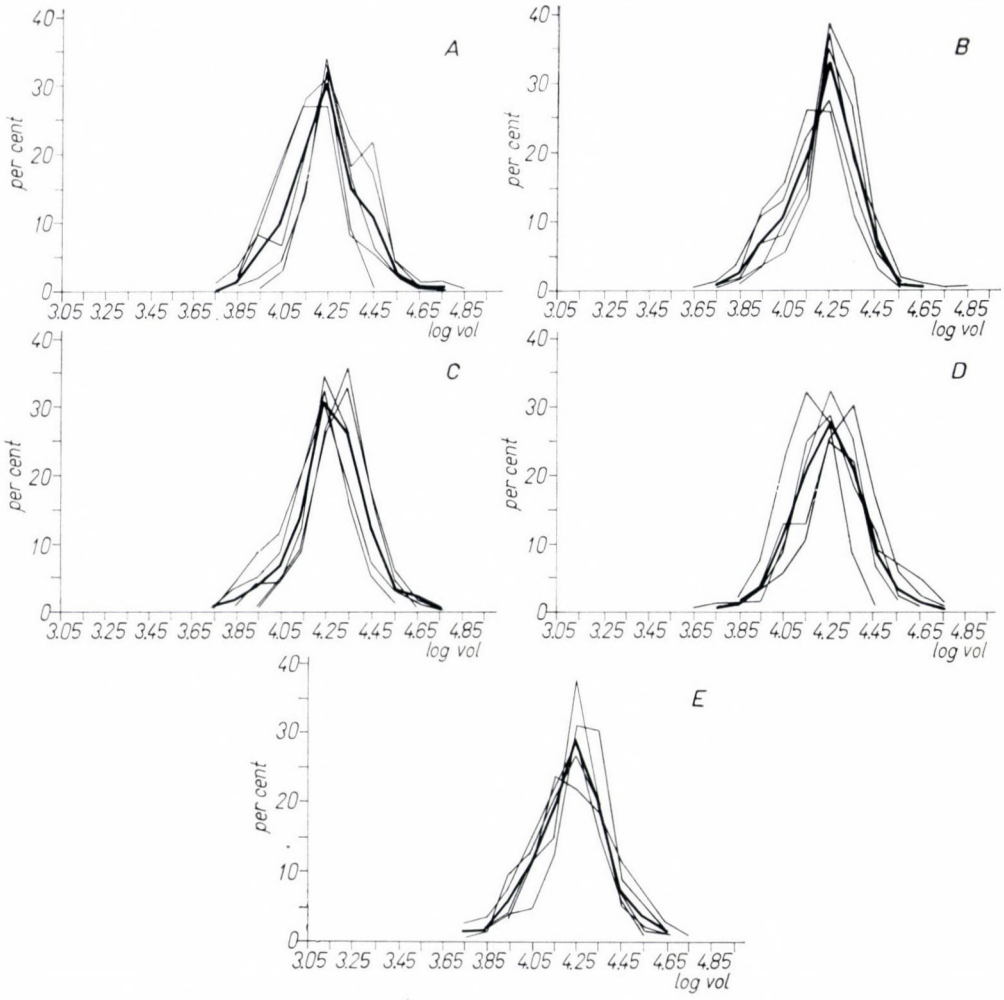

Fig. 3. Distribution curves of the average volume of the renal glomeruli
A = 11 weeks old C = 13 weeks old E = 15 weeks old
B = 12 weeks old D = 14 weeks old
For labelling see Fig. 1

In the group of the *four weeks old* animals the marked glomerular growth that started during the second week becomes even more intensive. This growth from the third till the fourth week was found to be 37.9%. In this period the glomeruli become more than twice as large as they were at birth. Parallel with

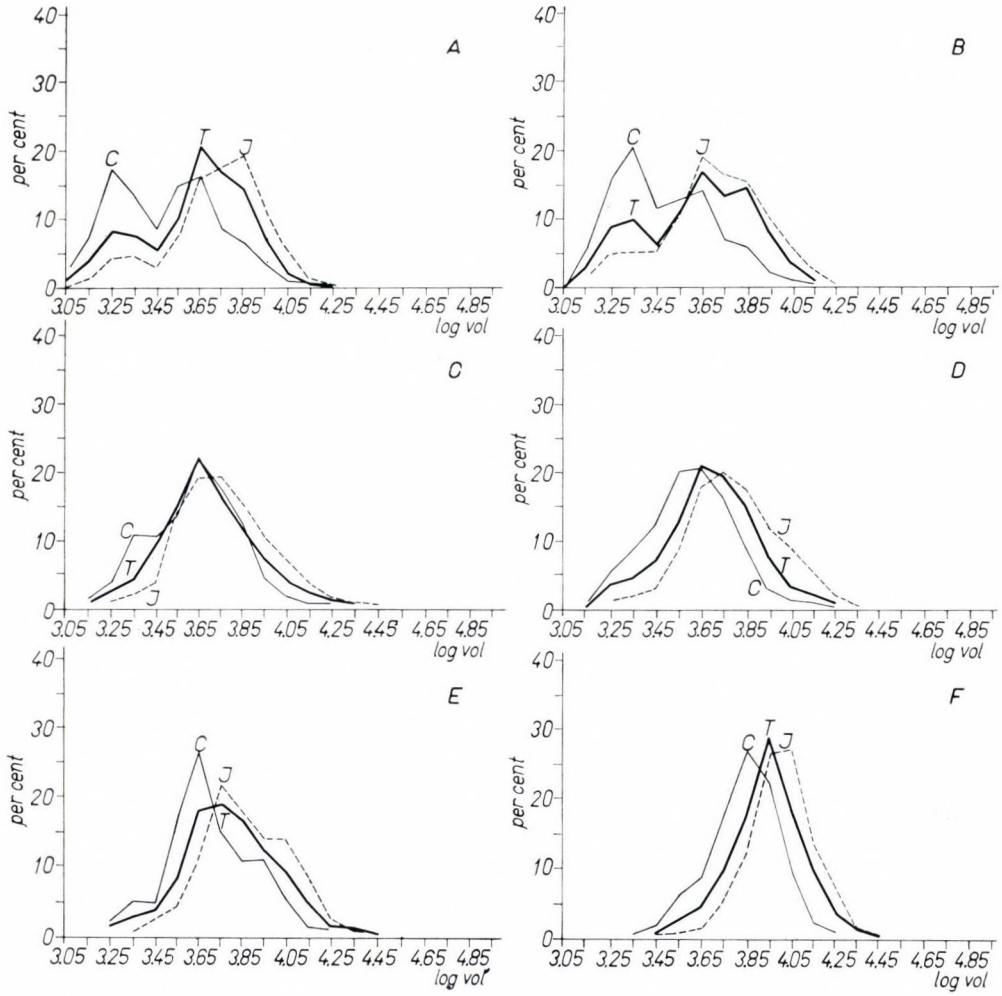

Fig. 4. Distribution curves of the volume of the glomeruli measured in the cortical and juxta-medullary zones and in the total cortex. Each curve represents the average value obtained from animals of the same age

T = glomeruli measured in the total cortex
C = glomeruli of the cortical zone
J = glomeruli of the juxta-medullary zone
For labelling see Fig. 1.

A = newborn
B = 2 days old
C = 1 week old
D = 2 weeks old
E = 3 weeks old
F = 4 weeks old

the growth, the glomeruli become more uniform in size, as it is indicated by the steep distribution curve (Fig. 1/f). Both the cortical and juxta-medullary curves are regular Gauss curves, neither a plateau nor more than one peak can be found. (Fig. 4/f.) As a result of the growth, the curves shift to the right. The difference of 47.1% between the glomeruli of the cortical and juxta-

medullary zone is still very wide. It is surprising that although the rate of the growth in size was the highest during these seven days, it was this week that saw the sharpest decline in the percentage distribution of the glomeruli in

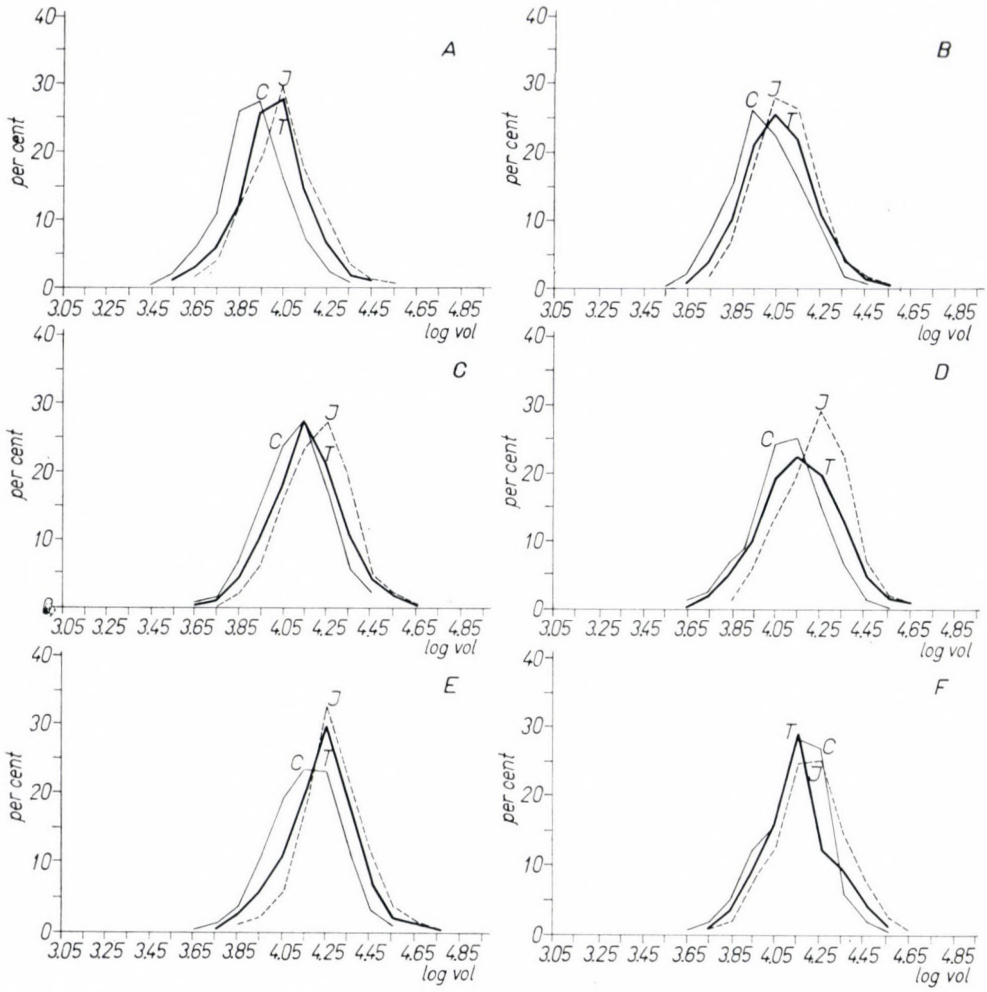

Fig. 5. Distribution curves of the volume of the glomeruli measured in the cortical and juxta-medullary zones and in the total cortex. Each curve represents the average value obtained from animals of the same age

T = glomeruli measured in the total cortex
C = glomeruli of the cortical zone
J = glomeruli of the juxta-medullary zone
For labelling see Fig. 1

the cortex. From a level of 6.14% of the previous week it went down to 4.35%, a value that comes fairly close to that of an adult animal and the rate of decrease during the period that followed was minimal. This indicates that in

the fourth week the growth of the tubules was very intensive, well exceeding that of the glomeruli which also increased in size at a high rate. The greatest change in the growth of the kidney of rats seems to take place during the fourth week.

In the group of the *five weeks old* animals this growth continues but at a slower rate than before. The glomeruli are uniform and the curves obtained are steep (Fig. 3/a). The difference between the cortical and juxta-medullary glomeruli is less marked as indicated by Fig. 5/a.

In the group of the *six weeks old* animals the rate of growth is similar as in the previous group, with the volume of growth of the glomeruli is 15.5%. By this time the glomeruli have grown almost three times larger than they were at birth, and their percentage distribution in the cortex has gone down to 4.5%. The glomeruli are evenly distributed as indicated by Figure 3/b, the juxta-medullary glomeruli are 22% larger than the cortical ones (Fig. 5/b).

In the group of the *seven weeks old* animals the final intensive rate of glomerular growth can be observed. As compared with respective figures in the previous week, the glomeruli have grown by 24.9%, which is an average growth of 3,000 μ^3. In this period the glomeruli of some animals used have reached their maximum size. After this period no change can be observed in glomerular size in the animals investigated. Also the percentage distribution of the glomeruli in the cortex is similar. During the period between the 7th and 15th weeks the distribution curves are regular and steep (Figs 2/c—f, 5/c—f, 3 and 6).

The glomeruli of the adult (from 10 weeks of age on) rats are four times larger than those of the newborn ones. The cortical glomeruli are 20% smaller in average than the glomeruli of the juxta-medullary zone. (Cortical: 14,000—15,000 μ^3, juxta-medullary: 17,000—19,000 μ^3; average: 16,000—18,000 μ^3.) The percentage distribution of the glomeruli in the cortex stands below 4% (3.66—3.76%).

Discussion

The principal aim of our glomerulometric analysis was to demonstrate the process of growth as well as the rate development of the glomeruli, and to investigate the relationship between the growth of the glomeruli and the growth of rest of the renal cortex.

On the basis of these investigations the following can be stated:

Changes in glomerular size

The growth of the glomeruli is uneven during the periods of various age groups investigated in the kidney of rats. The fact that the most intensive growth takes place between the 2nd and 7th weeks corresponds to that what has been detected by ARATAKI. During this period the volume of the glomeruli

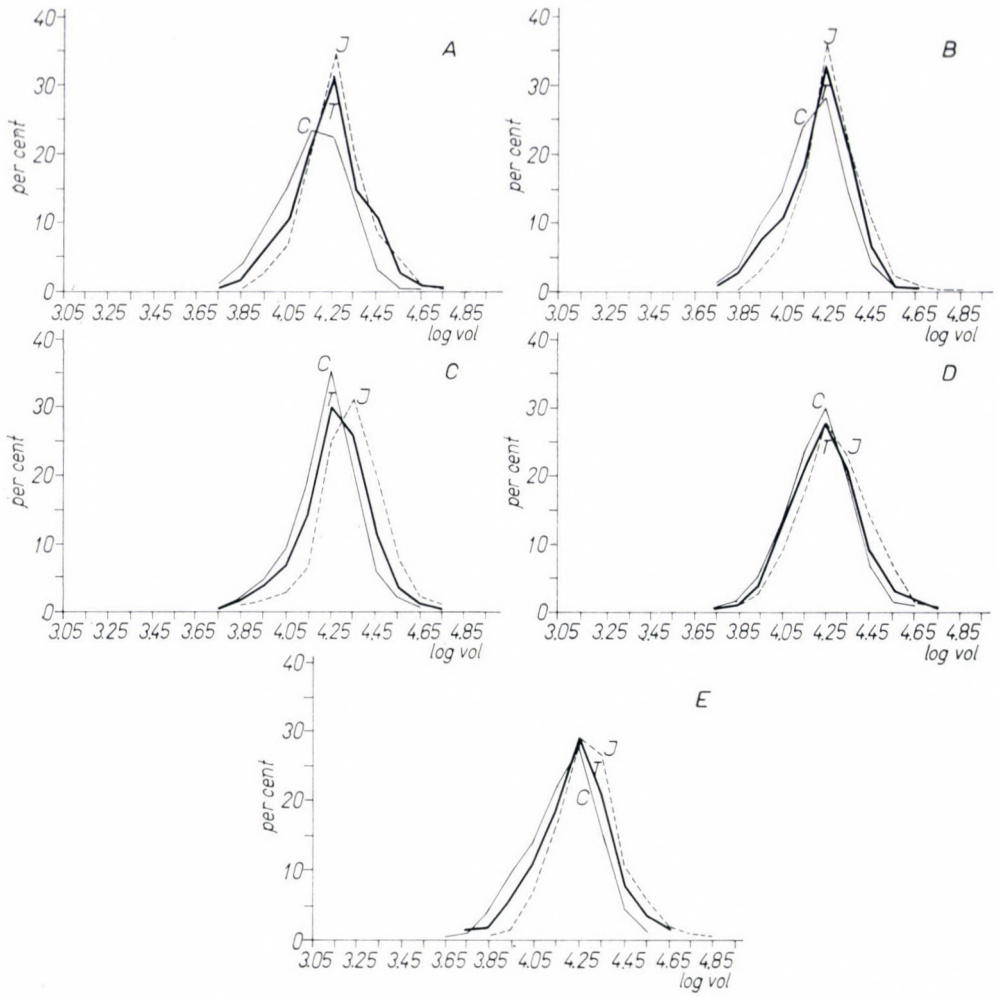

Fig. 6. Distribution curves of the volume of the glomeruli measured in the cortical and juxta-medullary zones and in the total cortex. Each curve represents the average value obtained from animals of the same age

T = glomeruli measured in the total cortex
C = glomeruli of the cortical zone
J = glomeruli of the juxta-medullary zone
For labelling see Fig. 1

A = 11 weeks old
B = 12 weeks old
C = 13 weeks old
D = 14 weeks old
E = 15 weeks old

increases three times in size. In the period of sucking the rate of growth is slower, but in contrast to ARATAKI's observations we could not observe any stagnation in growth. After the tenth week no essential change or growth occurred (Fig. 8). Comparing the relation of the glomerular size with the body weight and kidney weight we have found there parallel increasing with age

within some minor fluctuations. There is a deviation from this trend during the 7th week when the weight of the kidney increases considerably but neither the size of the glomeruli nor the body weight grows proportionately (Fig. 9).

A large number of small glomeruli can be detected in the new born group. Their size differ remarkably from those of the majority. The postnatal period

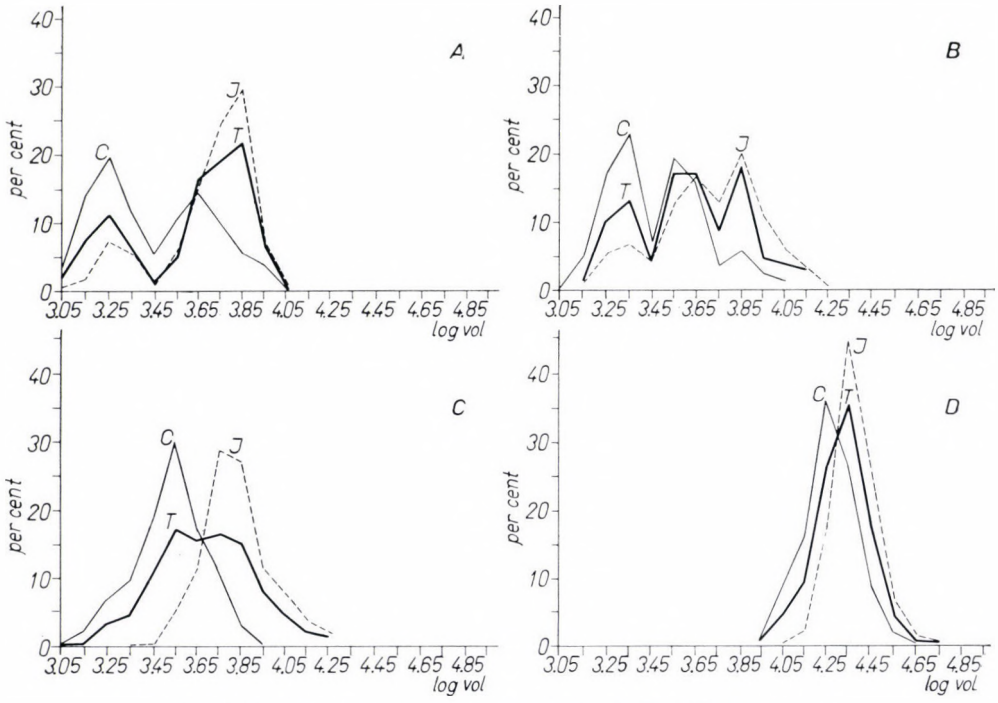

Fig. 7. Distribution curves characteristic of the different ages

A = newborn
B = up to second week after birth
C = 3 to 4 weeks old animal
D = adult animal
C = glomeruli of the cortical zone
J = glomeruli of the juxta-medullary zone
T = glomeruli measured in the total cortex

is characterized by several peaks on the curve (Fig. 7/a and 7/b). Later these small glomeruli become similar in size to the larger ones, and the distribution curve shifts to the right and becomes steep as being the results of growth and uniform size of the glomeruli. This feature is characteristic of the kidney of the grown up rats. The well discernible margin between the steep curves is due to the fact that the relations between the juxta-medullary and cortical curves are different. Provided the difference between the two types is reduced, then the two regular curves come closer to each other and, as a result, the average curves become regular and fairly steep Gauss curves (Figs 7/c and 7/d).

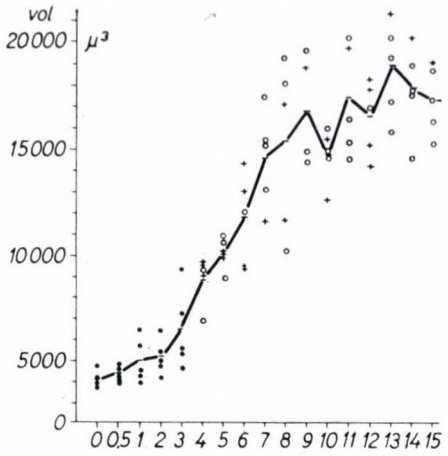

Fig. 8. Average of the renal glomeruli according to age
+ = male ● = female ——— = average

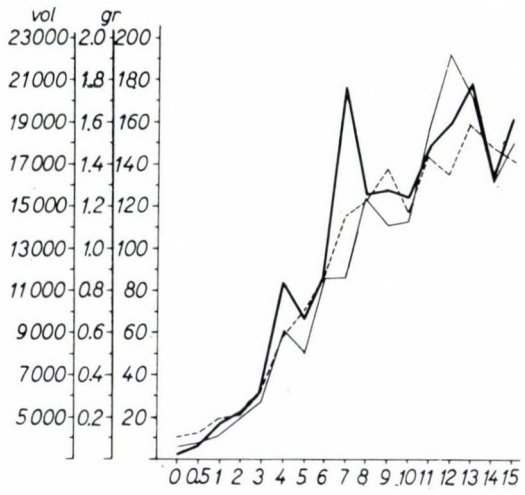

Fig. 9. Changes in the volumes of the glomeruli (- - - -), according to kidney weight (———) body weight (———) and age

The glomeruli of the juxta-medullary zone

In each kidney the cortical and juxta-medullary glomeruli were separately examined. At the various stage of age examined a distinct difference could be found; in each group of animals the glomeruli of the juxta-medullary zone were found to be larger.

The difference in size between the glomeruli of the two zones gradually diminishes as the animals grow older (Fig. 10). By the 10th to the 15th week the difference gradually drops to about 25% from a maximum level of over 70% at birth.

It has to be considered that the juxta-medullary zone includes not only the so-called juxta-medullary glomeruli but cortical types also occur there in

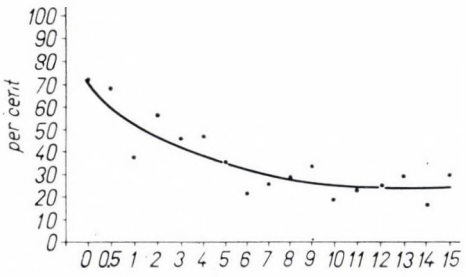

Fig. 10. Juxta-medullary — cortical ratio in percentage

large numbers. Accordingly, the juxta-medullary glomeruli proper are remarkably larger in size than the above given values. Information about their size can be obtained from an analysis of their distribution curve. The curve of the juxta-medullary glomeruli always shifts to the right by 1 to 3 classes as compared with the cortical curve; glomeruli of such a size cannot be detected in the cortical zone. Therefore it is very tempting to suppose that glomeruli of such a very large size correspond to the juxta-medullary glomeruli proper as described by TRUETA and co-workers [12]. The volume of these glomeruli is depending on age, they can be twice or even two and a half times larger than those of the cortical glomeruli.

The percentage distribution of the glomeruli in the cortex

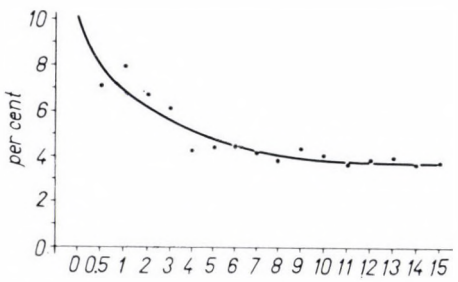

Fig. 11. Percentage distribution of the glomeruli in the cortex

The results obtained are identical with those found in literature. The ratio between glomerular volume and total tissue volume gradually decreases

with ageing. The highest value (10.1%) was found in new-born rat; during the first weeks after birth the ratio decreased rapidly and then the process slowed down. The ratio stood at 3.7% in the fifteenth week after birth (Fig. 11). It follows that however fast the glomerular growth is, the rest of the parenchyma in the cortex grows even faster.

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GLOMERULOMETRISCHE UNTERSUCHUNGEN II. EIN BEITRAG ZUM PROBLEM DES WACHSTUMS DER GLOMERULI BEI DER RATTE

Größe und Anteil der Glomeruli an der Rindensubstanz wurde an 85 männlichen und weiblichen weißen Laboratoriumsratten untersucht. Von der Geburt bis zum Alter von 15 Wochen wurden die Untersuchungen wöchentlich an Gruppen von je 5 Tieren durchgeführt. Dabei wurde auch der Größenunterschied zwischen den corticalen und juxtamedullären Glomeruli studiert. Das Wachstum der Glomeruli erfolgt nicht gleichmäßig. Nach der Geburt können viele Glomeruli beobachtet werden die kleiner sind, als der Durchschnitt, welche aber die Anderen nach der zweiten Woche einholen. Der Wachstum-Rhythmus ist bis zur zweiten Woche langsam, in der 2—7. Woche intensiv, von da an bis zur 10. Woche langsam und von diesem Zeitpunkt an kann kein nennenswerter Wechsel beobachtet werden. Die juxtamedullären Glomeruli sind immer größer, als die corticalen Glomeruli. Gleich nach der Geburt ist der Unterschied am größten (70%), nimmt dann schrittweise ab und beträgt in der 10—15. Woche nur noch 25%. Der Anteil der Glomeruli am Cortex beträgt bei der Geburt 10,1% und geht bis zur 15. Woche auf 3,7% zurück.

ГЛОМЕРУЛОМЕТРИЧЕСКИЕ ИССЛЕДОВАНИЯ. II. ДАННЫЕ К ВОПРОСУ РОСТА МАЛЬПИГИЕВЫХ КЛУБОЧКОВ У КРЫС

Авторы исследовали величины Мальпигиевых клубочков почек и их соотношения в корковой части. Исследование произвели на 85 белых крысах разного возраста и пола, от рождения до возраста 15 недель. Опыты были поставлены по неделям над группами,

состоящимися из 5 крыс. Цель исследования — изучить существует-ли различие в Мальпигиевых клубочках юкстамедуллярной и кортикальной зоны. Рост Мальпигиевых клубочков происходит неравномерно. После рождения можно найти очень много Мальпигиевых клубочков меньше среднего размера, которые после двух недель выравниваются с другими по размеру. Темп роста до 2-ой недели медленный, между 2—7 неделями интенсивный, между 7—10 неделями замедлен, а после 10 недель существенных изменений не обнаруживается. Мальпигиевые клубочки юкстамедуллярной зоны всегда больше по величине чем Мальпигиевые клубочки кортикальной зоны. Разница самая большая непосредственно после рождения (70%), которая постепенно снижается и к 10—15 недели составляет лишь 25%. Соотношение Мальпигиевых клубочков в коре при рождении — 10,1% и к 15 недели снижается до 3,7%.

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Acta Biol. Hung., **15**(4), 409—423 (1965)

GLOMERULOMETRICS III.

DATA REFERRING TO THE GROWTH OF THE GLOMERULI IN MAN

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(Received August 27, 1964)

Synopsis

Volumetric analysis of the glomeruli and their percentage distribution within the cortex has been examined in 67 men of different age groups and of both sexes in order to detect the changes in size and distribution of the glomeruli during the span of life. The glomeruli were taken as ellipsoids and their long and short axis was measured at a magnification of 250 times in order to compute their volumes. The percentage distribution of the glomeruli within the cortex was also determined. The difference in size between the glomeruli of the cortical layer and those of the juxta-medullary zone has also been examined. The glomeruli have been found to be increasing in size up to the age of about 30. This increase is most intensive between the 10th and 20th years of age. After 30 years of age the glomeruli become smaller and they are of varying sizes. The glomeruli of females are smaller than those of males. The juxta-medullary glomeruli are always larger than those of the cortical zone. This difference in size is the biggest after birth (50%), then it goes down to 20% during the rest of life. The percentage distribution of the glomeruli in the cortex drops to 6% between the 20th and 30th year of age comparing them with the postnatal level of 9%, and with the 5% in advanced age.

Introduction

A change in size of the glomeruli is a familiar symptom in some renal diseases. This change can be an increase or decrease in size or both concerning the various glomeruli within one kidney.

In a previous paper [13] we have published data referring to the growth of the glomeruli in rats. On the same principles we examined the changes in size of the glomeruli in man. Since various age groups were examined, it was possible to observe the effect of aging and the process of involution in this relation.

According to the literature [1] all glomeruli are to be found in man at the time of birth and they do not increase in number later; TOLDT [11], however, detected the formation of new glomeruli eight days after birth. The data referring to growth are rather contradictory. ECKHARDT [1] found glomeruli fully developed in size in a 32 year old man and MOBERG [7] assumed that the period of growth comes to an end between 35 and 40 years of age.

Results obtained by the measurement the cortical and juxta-medullary glomeruli are also contradictory. According to KÜLZ [6] at birth the glomeruli lying closer to the medulla are much larger than those near the surface. The rate of growth of the latter is faster and from the age of 2 both types of glomeruli are identical in size. MORISON [8] found in corrosion preparations that the glomeruli situated closer to the medulla were larger. MOBERG [7] described a difference in size between the two types of glomeruli only in the first months after birth while this difference diminishes after one year of age. Similar results were shown by TRUETA and co-workers [12]. MÖLLENDORFF [9] referred that there is no difference in size between the two types of glomeruli, but GLADSTONE [2] maintained the view that a difference in size as well as in form could be detected. According to HEIDENHAIN [4] the large juxta-medullary glomeruli are to be considered the first developing ones and they are the first to come into action, while the cortical glomeruli are in an embryonic stage at birth. HEGGIE [3] stated that 15% out of the total number of the glomeruli are juxta-medullary ones.

We have not found any data in the literature dealing with the influence of sex on the size of the glomeruli.

In a newborn the density of the glomeruli in the external cortex is 5 times more, and in the juxta-medullary zone twice more then that is in an adult. Similar data have been published by MOBERG [7]. According to KITTELSON [5] the percentage distribution of the glomeruli in the cortex is 4.76%, whereas GLADSTONE [2] states that it stands at 6.6%. The percentage distribution decreases with ageing [1].

Material and method

Kidneys of 67 men of different age groups and of both sexes were used (Table 1). Autopsies were obtained from the Department of Forensic Medicine, Med. Univ. Budapest. taken from persons who had died in accidents and in their reports available there were no renal diseases and by histologic examinations the kidneys proved to be normal or adequate to age. Transverse sections from the kidneys were fixed in formalin (4%) then embedded in paraffin-wax, cut at 17 μ and stained with haematoxyline-eosin. Diameters of 300 glomeruli were measured at random on sections from each specimen and than on the slices the juxtamedullary and cortical zones were separated under a stereo microscope by a line of China ink drawn on the glass cover. An additional 300 measurements were made in each separated zone. Measurements were made at a magnification of 250 times as described in our previous papers [10, 13]. The volume of the glomeruli has been computed in each zone and represented in a distribution curve. The average volume of the glomeruli was determined in μ^3.

The percentage distribution of the glomeruli in the cortical layer was measured by our method described in a previous paper [10].

Results

From birth to one year of age the size of the glomeruli is only a tenth of that of an adult. Their size ranges from 12,600 μ^3 to 41,600 μ^3 according to age; the average is 21,000 μ^3 (Fig. 1/a). Within these limits various glomerular volumes can be observed in the entire cortex. The distribution curve is flat and broad as seen in Figure 3/a. Several peaks or plateaus occur on the curve. Small glomeruli can be found in both the cortical and juxta-medullary zones, and presumably that is why the difference in size between the juxta-medullary and cortical glomeruli is only 36.5%. This value indicates that at this age the difference in glomerular size between the two zones is not the biggest. The ratio of the glomeruli in the cortex is also the highest at this stage with a total of 8.69%, a level which lays behind that of the rat.

From one to ten years of age a 70% glomerular growth takes place, a figure well below of that which follows during the development of the subsequent years. Therefore the distribution curve is shifted to the right, but it remains to be flat.

There is a considerable difference, ranging from 6,000 to 120,000 μ^3, between the volumes of the glomeruli. The growth of the juxta-medullary glomeruli is more intensive, than that of the cortical ones. The difference between the two zones is 51.7%. The percentage distribution of the glomeruli is 8.6%, this indicates that there is no essential change in this relation in this age (Figs 1/b and c, and 3/b and c).

Between 11 and 20 years of age the glomeruli increase in size significantly; their growth exceeds that of the preceding decades seven times; during this period the glomeruli grow ten times larger in size than they do in the year following the birth. They are only 10 to 30% smaller than those of an adult person. Their average volume ranges between 140,000 and 200,000 μ^3 from the age of 12 to 15, and between 230,000 and 260,000 μ^3 from 15 to 20 years of age. By this time the glomeruli have become even, the small glomeruli found after birth are no longer seen. The distribution curve is regular and steep with one peak. (Fig. 1/d.) The difference between the two zones is still wide, 39.2% as indicated in Figure 3/d. Although the growth of the glomeruli was very intensive, the rate at which the rest of the cortex grew was even higher; as a result the percentage distribution of the glomeruli in the cortex fell from 8.6% to 6.4%. It still stands at a higher level than in adults, which indicates that the growth of the glomeruli comes to an end earlier than that of the tubuli.

From 21 to 30 years of age a slight growth can still be observed and the average of the total glomerular volumes is 220,000 μ^3. On the distribution curve of the average glomerular volumes (Fig. 1/e) a plateau is seen. This is a result of the fact that a big difference can be found between the various cases and so the peaks of the various curves do not fall in the same class; if they are

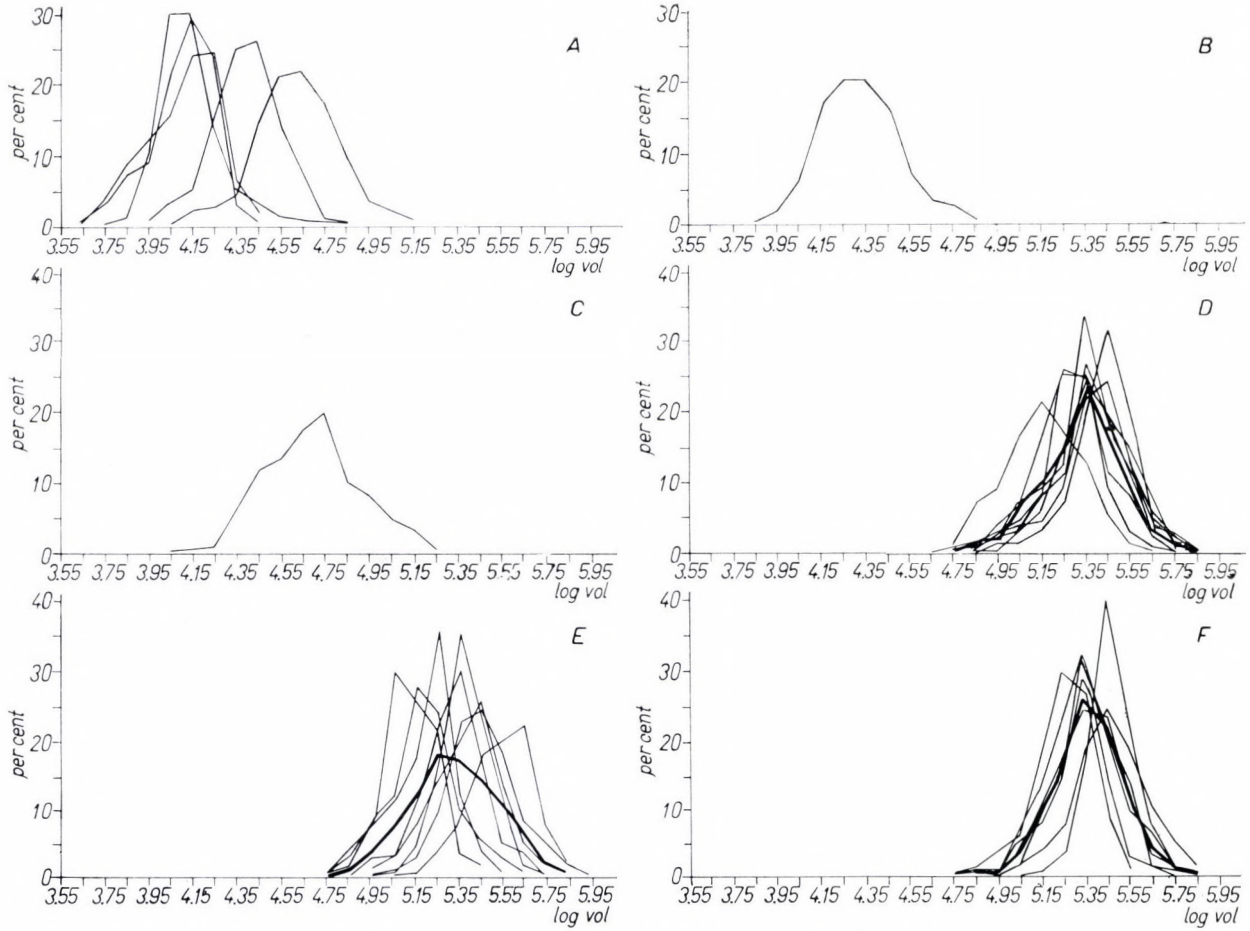

Fig. 1. Distribution curves of the average volume of renal glomeruli

A = 0—1 year C = 7 years E = 21—30 years
B = 3 years D = 11—20 years F = 31—40 years

The logarithms of the volume categories are presented horizontally; the number of glomeruli belonging to each category and expressed in percentage is given in the vertical line
The thick line represents the average of the glomeruli of individuals of the same age

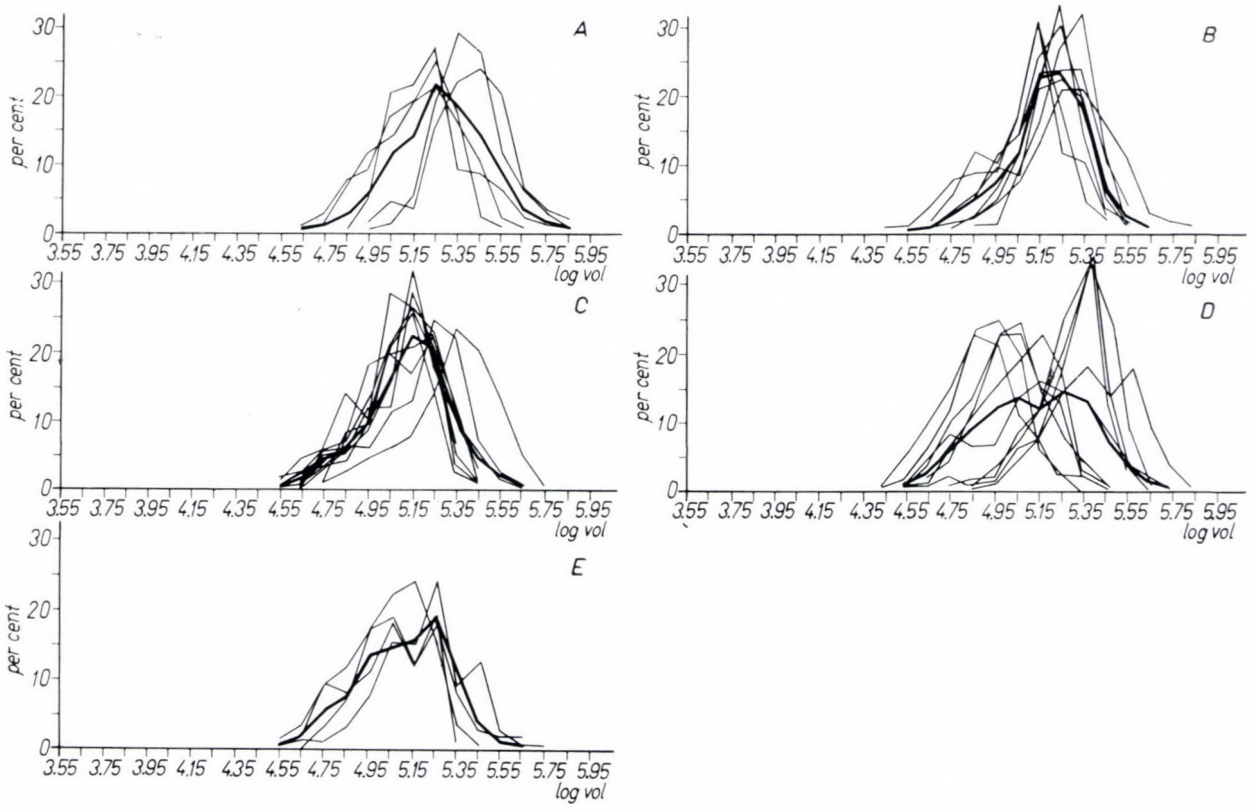

Fig. 2. Distribution curves of the average volume of the renal glomeruli

A = 41—50 years C = 61—70 years E = 81—90 years
B = 51—60 years D = 71—80 years

Legend see also Fig. 1

Table 1

Age group in years	Age	Sex	Average glomerular volume in entire cortex		Average glomerular volume in cortical zone		Average glomerular volume in juxta-medullary zone		Difference in size between cortical and j. m. glom. in %	Percentage distribution of glomeruli in the cortex
			μ^3	average of group	μ^3	average of group	μ^3	average of group		
day	2	+	13826		12030		15787			
month	3	○	12772		11377		14757			
month	3	○	12647		11032		17220		36.46	8.69
month	4	+	24905		23385		27670			
year	1	+	41650		36840		54150			
	3	+	21025		17970		25510		57.75	8.60
	7	○	48570		37820		61100			
	12	○	143420		119200		177000			
	13	○	199550		173500		274400			
	15	+	181130		168250		236900			
11—20	17	+	266900	217120	258600	199119	288200	275600	39.23	6.36
	18	+	237300		221300		296700			
	19	+	238400		219450		290400			
	20	+	241550		227900		312800			
	20	+	228700		204800		330400			
	21	○	131700		121550		137400			
	21		213800		207000		222450			
	22	○	152200		145067		156667			
21—30	24	○	155700	220518	143760	214503	164450	241490	12.22	6.12
	28	+	256450		237850		314500			
	29	+	239150		228700		252200			
	30	+	349150		330800		390500			
	30	+	283800		282700		293750			
	32	+	280300		255050		363600			
	35	+	196950		188630		220600			
	35	+	180130		174030		188550			
31—40	36	+	232950	234967	209550	219330	268900	266314	20.58	6.38
	36	+	231000		214950		264850			
	37	+	292400		279000		315200			
	37	+	231050		217100		242500			

41—50	41	+	139830	194096	130733	191625	148470	208848	13.96	6.07
	42	○	169400		163533		179170			
	43		153200		155830		149500			
	46	+	249050		208750		293500			
	50	+	259000		247750		273500			
51—60	52	+	198500	150294	192660	145222	208950	165796	17.45	5.84
	52	○	120233		103525		147430			
	54	+	150200		141800		151100			
	54	○	119125		93190		139625			
	56	+	146400		129200		148267			
	56	+	170500		170233		177700			
	57	+	187500		179567		189200			
	59	+	157900		157600		164100			
61—70	61	○	131225	140178	124100	134020	137500	147253	10.24	5.62
	62	+	126967		125900		130800			
	63	+	133867		127825		141033			
	64	+	140066		138433		143467			
	65	+	153470		135530		171800			
	66	+	148100		123325		177530			
	67	+	108575		108380		105750			
	67	+	215650		217100		218750			
	68	○	115267		114200		111625			
	69	+	128600		125400		134275			
71—80	71	○	221850	144945	219450	135636	228700	159770	19.68	5.05
	71	○	186650		177250		195000			
	74	○	230450		204130		255250			
	74	○	84060		82420		102500			
	74	+	110900		104900		129000			
	76	+	108525		104000		114100			
	78	○	133730		114900		162830			
	78	+	196600		189500		215450			
	79	○	97350		92050		109225			
	79	+	76340		67760		85640			
81—90	81	○	156667	125610	137700	117048	193050	135718	14.27	5.08
	84	○	123125		115075		130650			
	86	○	102800		103450		103850			
	89	+	120150		111967		115325			

averaged, a plateau can be obtained. The curves of the cortical and of the juxta-medullary glomeruli are approximately regular Gauss curves. Analysis of the glomerular volumes revealed that the difference between males and females is very considerable in this relation particularly in this age group. The average volume of the glomeruli in males is 264,750 μ^3 and that of the females is 146,500 μ^3. The differences between the sexes can mostly be observed during the 21 to 50 years period, and they level up by old age. The difference between the glomeruli of the juxta-medullary and cortical zones is less in this age group and there is no essential change in the period that follows either, but the juxta-medullary glomeruli always exceed those of the cortical zone in size (Fig. 3/e). The percentage distribution of the glomeruli in the cortex continues to decrease and stands at 6.12%.

The largest glomerular volumes can be found *between 31 and 40 years of age*. The average volume of the glomeruli is 235,000 μ^3. In this age group the glomeruli are the most even, their distribution curve is regular and steep as indicated in Figure 1/f. A wide margin between the two sexes is still to be detected and a difference of 20% can be observed between the two zones of cortex. There is no change whatever in the percentage distribution of the glomeruli. Relying on this it is very likely that the structure of the kidney is stabilized during this period and further alterations may indicate either ageing or some pathological conditions.

The glomeruli to be found in *the 41 to 50 years of age* group are smaller in size than those of the previous group. The average volume of the glomeruli is 194,000 μ^3. However, there is still a remarkably wide difference between the values of the two sexes and between the individual cases within one sex, but this is much greater than in the preceding age groups (Fig. 8/a). The difference between glomerular volumes of the two zones or percentage distribution of the glomeruli in the cortex is similar to that of the previous group with a declining tendency in both cases (Fig. 4/a).

During the period between 51 and 60 years of age the average volume of the glomeruli continues to fall to a level of 156,300 μ^3. The most important change here, in contrast to the subsequent age groups, is that there are no very small glomeruli within the range of volumes but there seems to be an overall decrease in volumes in all kidneys within this group. Therefore the decrease of the average volumes is due to the latter; they are 200,000 μ^3 in all cases. Within one kidney the glomeruli are still fairly uniform in size and their distribution curve continues to be regular but there is already a shift to the left as compared with the previous age groups (Figs 3/b, and 4/b). In this group the percentage distribution of the glomeruli in the cortex attains an average of 5.84%.

From 61 to 70 years of age the volumes of the glomeruli continue to decrease and the average is 140,200 μ^3. In certain cases a small plateau can be ob-

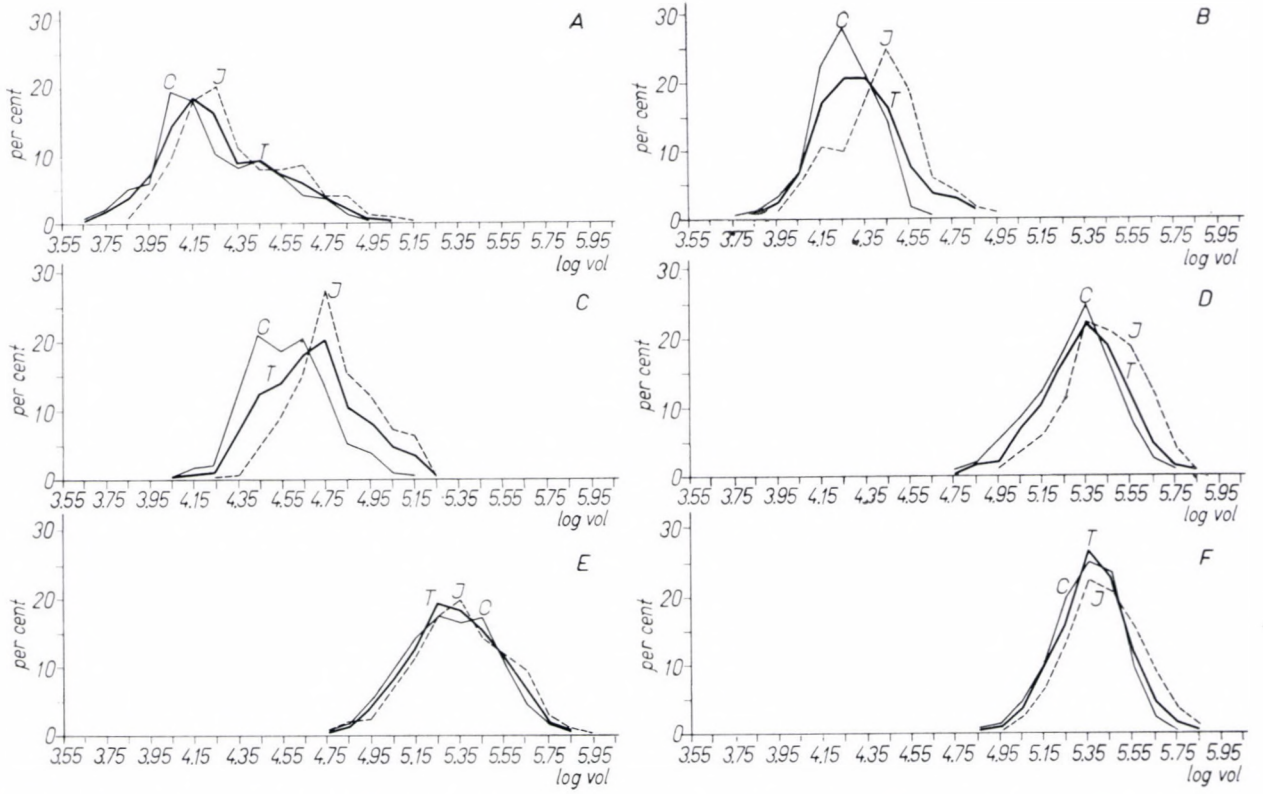

Fig. 3. Distribution curves of the volume of the glomeruli measured in the cortical and juxta-medullary zones and in the total cortex

T = glomeruli measured in the total cortex; Y = glomeruli of the juxta-medullary zone; C = glomeruli of the cortical zone
A = 0—1 year B = 3 years C = 7 years D = 11—20 years E = 21—30 years F = 31—41 years
Legend see also Fig. 1
Each curve represents the average curves of persons of the same age group

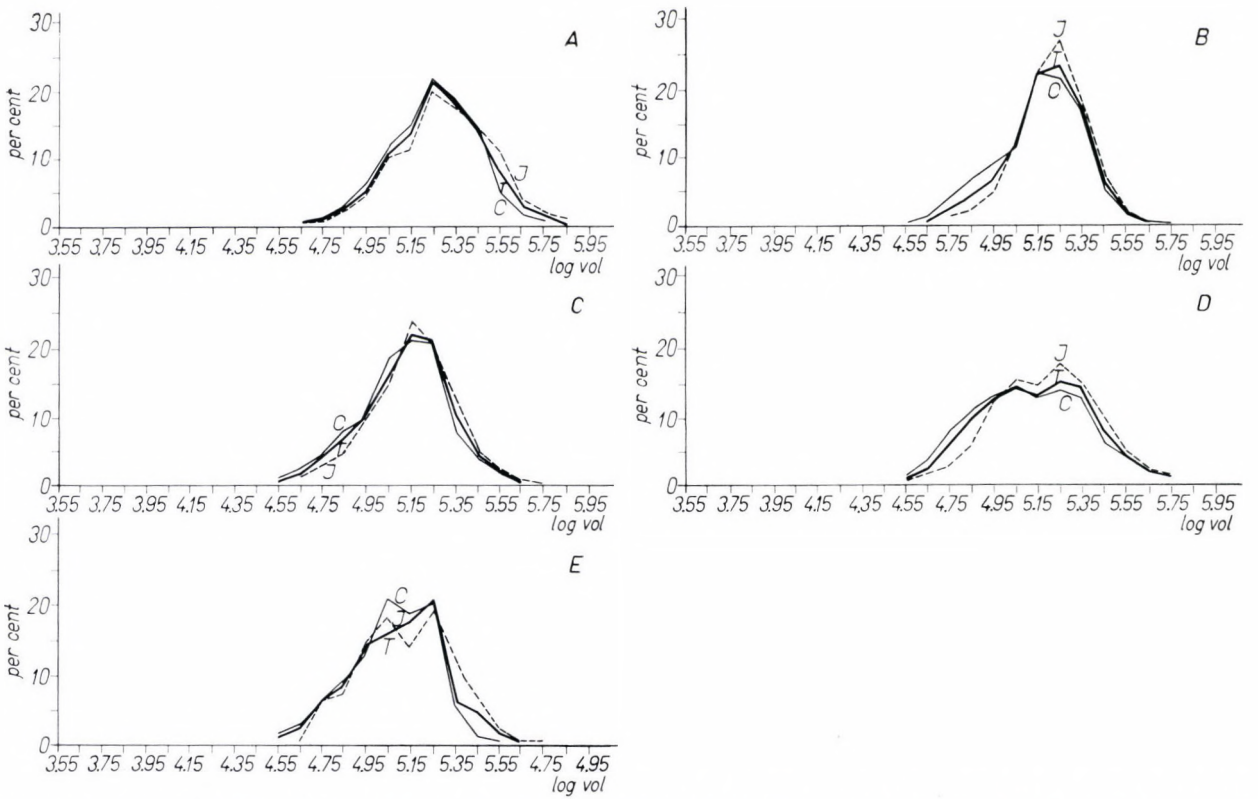

Fig. 4. Distribution curves of the volume of the glomeruli measured in the cortical and juxta-medullary zones and in the total cortex

T=glomeruli measured in the total cortex; Y=glomeruli of the juxta-medullary zone; C=glomeruli of the cortical zone
A = 41—50 years B = 51—60 years C = 61—70 years D = 71—80 years E = 81—90 years

served on the left side of the distribution curve, indicating the occurrence of small and presumably sclerotic glomeruli (Fig. 3/c). The idea that this is a change characteristic of old age is verified by the fact that in the subsequent age groups this process becomes more intensive, small glomeruli considerably increase in number, therefore the plateau is replaced by a separate peak.

From 71 to 80 years of age curves with two peaks could be obtained as referred earlier, in both the cortical and juxta-medullary zones (Fig. 4/d). The curves are flat and a definite shift to the left can be seen, but it is rather like a stretch, which means that large glomeruli can still be found in great numbers, in some cases even more than in the previous groups, which appears to be compensatory for the small sclerotic glomeruli (Fig. 3/d). The percentage distribution of the glomeruli also shows changes because of the intensive process of decrease in number of the glomeruli. Since the average volume of the glomeruli declines only to a lesser extent, and an increase in the number of the tubuli is out of question, it is presumable that at this stage a degeneration of the glomeruli has to be taken into account.

From 81 to 90 years of age the average volume of the glomeruli stands as low as 125,600 μ^3. The distribution curve shows two peaks. The curves are flat and irregular, indicating the presence of a number of glomeruli of different sizes (Figs 2/e and 4/e). The average volume of the juxta-medullary glomeruli is higher by 14.2% than that of the cortical ones. The percentage distribution of the glomeruli in the cortex is 5.1%.

Discussion

Based on these results the following can be said about the changes in the size of the glomeruli in the human kidney:

Changes in the volume of the glomeruli sections

In man growth of glomeruli can only be observed until 30 years of age. The rate of growth in the first years is comparatively slow. An intensive development is taking place after the 10th year. During the period of puberty the glomeruli grow six or seven times bigger and their size amounts from 70 to 90% those of an adult. The rate of growth slows down between 20 and 30, and a gradual decrease can be detected after 30. The decline in volume, however, is uneven, different values are obtained particularly in advanced age, and besides the small sclerotic glomeruli, those of a normal size or even larger ones can be found. After 30 years of age the volume curve shows a descending trend (Fig. 6) which, apart from some oscillation, can be followed up to the age of 90.

When the material is classified according to sexes, we find that in the youth the glomeruli of the male's kidney are some 20 to 40% larger than those

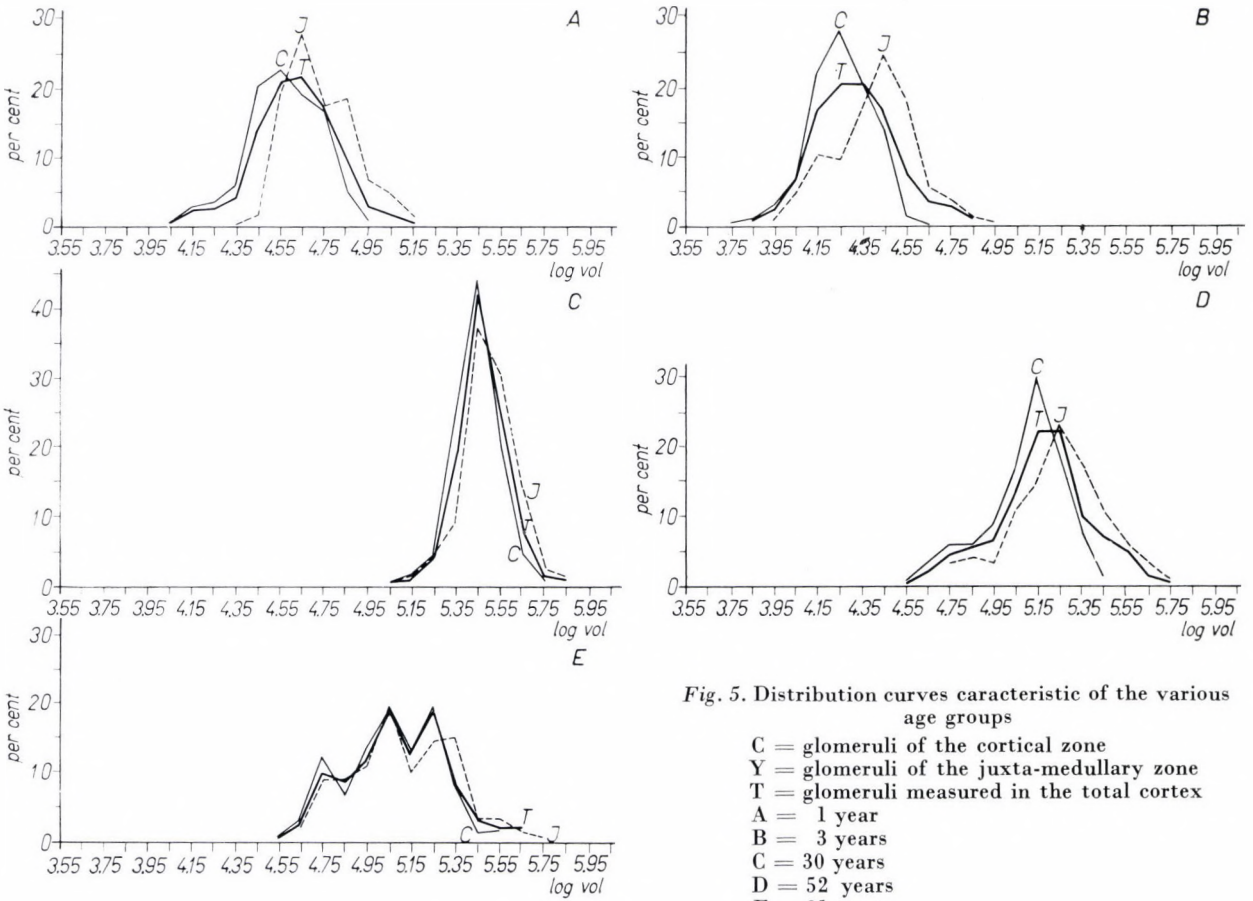

Fig. 5. Distribution curves caracteristic of the various age groups

C = glomeruli of the cortical zone
Y = glomeruli of the juxta-medullary zone
T = glomeruli measured in the total cortex
A = 1 year
B = 3 years
C = 30 years
D = 52 years
E = 81 years

of the kidney of females. In females the maximum level of growth is reached earlier (at the age of 15), and glomeruli practically remain to be of the same size until 40, but they are smaller than those of males throughout the whole period examined. The decrease in the volume, so characteristic of the male's glomeruli, can hardly be detected with those of the females.

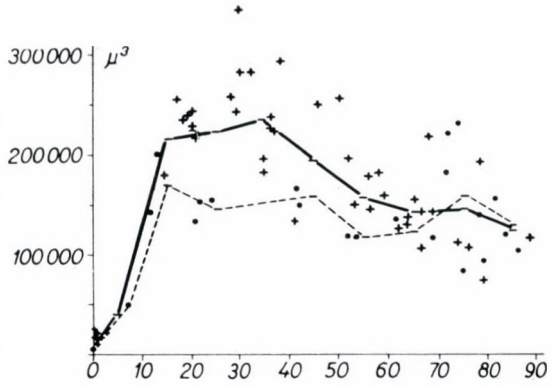

Fig. 6. The average volume of the glomeruli with respect to age
+ = male
● = female
——— = the average of the glomeruli of kidneys measured
- - - - - - - = the average of the glomeruli of the kidneys of females

The glomeruli of the juxta-medullary zone

In the human kidney the glomeruli found in the juxta-medullary zone are always larger than those in the cortical zone. The widest difference can be observed during the postnatal period (Fig. 7) when the juxta-medullary

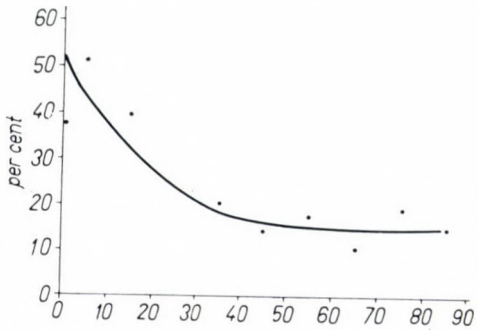

Fig. 7. The juxta-medullary — cortical ratio expressed in percentage

glomeruli exceed their counterparts in the cortical zone by about 50%. In the course of growth the margin gradually narrows down to an average of 20% in adult age. The changes taking place in the volume of the glomeruli in ad-

vanced age effect the glomeruli of both zones and this can be seen from the double peaks of the distribution curves of both zones.

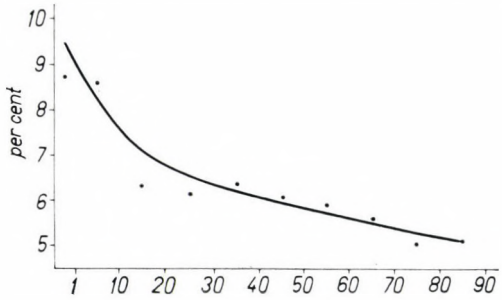

Fig. 8. The percentage distribution of the glomeruli in the cortex

The percentage distribution of the glomeruli in the cortex

The percentual proportion of the glomeruli in the cortex gradually decreases during the span of life. This corresponds to data published by ECKHARDT [1], KITTELSON [5] and GLADSTONE [2]. After birth the glomeruli account for almost 9% of the cortex. By the period from 15 to 25 years of age this ratio falls to 6% and finally it stands at as low as 5% in old age as indicated in Figure 8. The proportion of the glomeruli to the parenchyma declines in spite of the fact that their volume continues to grow and there is no change whatever in their number. This means that the rate at which the rest of the kidney, particularly the tubuli grow, well exceeds the rate of growth of the glomeruli.

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GLOMERULOMETRISCHE UNTERSUCHUNG III. EIN BEITRAG ZUM PROBLEM DES WACHSTUMS DER GLOMERULI BEIM MENSCHEN

An 67 verschieden alten Männern und Frauen wurde die Größe der Glomeruli und die Änderung ihres prozentuellen Anteiles an der Rindensubstanz während des Lebens untersucht. Bei 250facher Projektionsvergrößerung wurden die Durchmesser der Glomeruli gemessen und danach ihr Volumen berechnet. Gleichzeitig wurde ihr prozentueller Anteil an der Rindensubstanz bestimmt. Es wurde auch der Größenunterschied zwischen juxtamedullären und corticalen Glomeruli untersucht. Die Größenzunahme der Glomeruli dauert bis zum 30. Lebensjahr und ist zwischen dem 10. und 20. Jahr am intensivsten. Nach dem 30. Jahr werden die Glomeruli kleiner und die Größenunterschiede zwischen ihnen deutlich. Glomeruli von Frauen sind kleiner als die der Männer. Die juxtamedullären Glomeruli sind immer größer als die corticalen. Der Unterschied ist nach der Geburt am größten (50%) und vermindert sich später bis auf 20%. Der prozentuelle Anteil der Glomeruli am Cortex beträgt bei der Geburt 9%, nimmt zwischen dem 20—30. Lebensjahr ab (6%) und erreicht im Alter den Wert von 5%.

ГЛОМЕРУЛОМЕТРИЧЕСКИЕ ИССЛЕДОВАНИЯ. III. ДАННЫЕ К ВОПРОСУ РОСТА МАЛЬПИГИЕВЫХ КЛУБОЧКОВ У ЧЕЛОВЕКА

Авторы исследовали в течение жизни изменение соотношений величин Мальпигиевых клубочков в корковой части почки у 67 трупов людей различающихся по возрасту и полу. При проекционном увеличении в 250 раз измеряли диаметры Мальпигиевых клубочков, из этого выясняли их содержимое в кубических сантиметрах, а также определяли сколько процентов Мальпигиевых клубочков содержит корковая часть почки. Цель исследования изучить разницу в величине Мальпигиевых клубочков юкстамедуллярной и кортикальной зон. Рост Мальпигиевых клубочков происходит до возраста 30 лет. Темп роста наиболее интенсивный между 10—20 годами. После 30 лет Мальпигиевые клубочки уменьшаются и различаются по величине. Мальпигиевые клубочки женщин меньше мужских. Юкстамедуллярные Мальпигиевые клубочки всегда больше по размерам чем Мальпигиевые клубочки кортикальной зоны. Эта разница самая большая непосредственно после рождения (50%) и в течение жизни она снижается до 20%. Соотношение Мальпигиевых клубочков в коре с 9% (после рождения) снижается к 20—30 году жизни до 6% и в дальнейшем до 5%.

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Acta Biol. Hung., **15**(4), 425—430 (1965)

STUDIES ON EXCRETION IN THE COMMON INDIAN LEECH, HIRUDINARIA GRANULOSA (SAVIGNY)

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(Received September 15, 1964)

Synopsis

With the help of a new technique, developed by the author, pure leech-urine, collected directly from the nephridia, has been analysed for the first time. Quantitative estimations of ammonia N, urea N, non-protein N (NPN), and total N in the urine and in the supernatant fluid of the centrifuged urine were carried out by the Conway's microdiffusion technique. Certain minor modifications have been suggested so far as cleaning of the Conway units is concerned. About 32% of the total NPN is excreted in the form of ammonia N, and 14% as urea N. Rest of the NPN is excreted mostly in the form of free amino acids. Size and appearance of the nephridial bladders; total yield of pure leech-urine; physical properties, microscopic appearance, pH, and water content of the urine were examined. Quantitative estimation of ammonia N, and urea N in the water, containing leeches, was also carried out for comparison. Effect of drought on the water content of the leech-urine was also observed.

Introduction

Studies on the excretory system of *Hirudinaria granulosa* have been confined to its structure and development [2]. No work has, however, been done on the physiology of excretion in these animals. HEIDERMANNS [13] collected the urine of *Hirudo medicinalis* by keeping the worms in water and analysed the latter for nitrogenous contents. However, no attempts have hitherto been made to collect, for analysis, the urine directly from the nephridia of the leeches. The present studies have been undertaken with a view to collect, and to analyse, the pure leech-urine.

Material and method

For collection of the excreta two methods were adopted. Leeches of approximately similar weight were selected, washed and kept for certain periods in distilled water in flasks. The water was afterwards analysed for ammonia N and urea N. Alternately, urine was collected from the nephridia directly. Usually leeches are dissected after narcosis in dilute alcohol. Due to excessive wriggling and tortuous movements during the process, the nephridial bladders became almost empty. Use of various other general anaesthetics did not increase the overall

yield of urine either. When the animals were quickly killed by using a solution of corrosive sublimate, the nephridial bladders were found tightly filled with urine presumably due to sudden closure of the nephridiopores, but this method made the animals difficult to dissect. It was found the best to dissect the leeches without narcosis. A mid-dorsal incision was made after quickly stretching the leeches. Blood from the crop was washed away under the tap. The terminal vesicles of the testicular nephridia were carefully exposed and washed again. Excess of water was blotted from the surface of the dissection and vesicular fluid was taken out by means of a syringe.

Size and appearance of the nephridial bladders; total yield of pure leech-urine; physical properties, microscopic appearance, pH and water-content of the urine were examined.

Quantitative estimations of ammonia N, urea N, non-protein N (NPN) and total N were carried out by Conway's [7] microdiffusion technique. Unit No. 1 was mostly used. Conway's method of cleaning the units was followed with minor alterations. After removing the excess of fixative, the units were kept overnight in petrol, washed with soap using a cotton pledget, treated with chromic acid (45 min.), washed thoroughly under the tap, rinsed with double distilled water to avoid inhibition of urease by traces of heavy metals, and dried in an oven. Several kinds of fixatives were tried for sealing the units. Resealing of the units was unsatisfactory with the water-soluble fixative although cleaning of the units was very convenient. It also contained appreciable quantity of ammonia, which could not be completely removed by prolonged bubling, and was, therefore, unsuitable for microestimations. Greases of high melting point worked well.

For estimating ammonia N at a concentration higher than about 15 μ g/ml, boric acid with indicator was used as an absorbent in the central chamber of the unit. Saturated solution of potassium carbonate was used for liberating ammonia in the outer chamber. Experimental fluid was delivered accurately by means of a simple tube pipette. The sealed units were kept on an oscillating table. After the completion of the diffusion period the contents of the central chamber were titrated against a standardized HCl solution from a Conway's microburette. Blanks were always carried out side by side.

For estimating ammonia N at a level lower than about 15 μ g/ml the $Ba(OH)_2$—HCl procedure [7] was adopted. The strength of the $Ba(OH)_2$ solution was accurately checked before a titration series, using a Conway's burette and an "Agla" micrometer syringe for delivering the alkali and the acid respectively. Neutralized distilled water was used for preparing and diluting the standard solutions.

For estimating total N a known volume of the urine was digested in a micro-Kjeldahl flask. Conc. H_2SO_4, $KHSO_4$, and SeO_2 were used for digestion. Incineration was carried out for 3—8 hours on a micro-Kjeldahl digestion stand. The cooled and diluted digest was analysed by Conway's microdiffusion technique. Total N was also estimated in the supernatant fluid of the centrifuged urine. For estimating NPN in the urine, ethanol was used for deproteinization. The supernatant fluid was digested and analysed.

Results

Size and appearance of the nephridial bladders

In a fully stretched and dissected leech the nephridial bladders or terminal vesicles looked like tiny beans. The vesicles of the pre-testicular nephridia were 0.20—0.35 cm long and 0.11—0.22 cm wide. The vesicles of the testicular nephridia were 0.35—0.65 cm long and 0.20—0.40 cm wide.

Total yield of pure leech-urine

About 15 ml of urine was collected from 150 leeches of various sizes. Exceptionally, however, as much as 0.4 ml and as little as 0.02 ml of urine was collected from a single animal.

Physical properties, microscopic appearance, pH, and water-content of the urine

The urine was turbid in appearance due to rich bacterial growth. When leeches were examined under a binocular microscope, only clear fluid devoid of the whitish matter was seen coming out of the nephridiopores. Bacterial cells were also seen in water in which the animals were kept submerged for some time. No macroscopic clumps were seen in the water, indicating that these are retained inside the bladders.

When the urine was kept in a tube for some time, most of the whitish matter settled down, and comprised 7—25% of the urine by volume. On centrifugation (4,000 r.p.m.) the supernatant fluid was found to be clear and colourless. The residual mass was either whitish or had a faint pinkish tinge.

Table 1

Quantitative estimation of ammonia N and urea N, voided in water by a leech — submerged for different periods

Duration in hrs.	Ammonia N in μg (Mean)	Urea N in μg (Mean)
12	1015.5	205.4
15	1434.8	27.5
21	972.7	334.1
24	1853.3	154.4
36	1223.5	394.4
48	1737.1	132.5
72	1513.8	338.9
144	1958.9	4.5

The urine was usually tasteless, but sometimes it was salty.

On the addition of bromocresol purple (sensitive to pH 5.2—6.8) the urine remained light purple. Addition of (B.D.H.) universal indicator produced a faint greenish yellow colour, indicating that its pH was between 7.0 and 7.5.

On drying at 100°C or desiccating over anhydrous $CaCl_2$ at room temperature, the urine yielded flakes of light brownish hue. Flaky mass of similar hue but lesser in proportion was obtained when the supernatant fluid of the centrifuged urine was dried or desiccated.

The solid matter in whole urine was 0.30—3.99% and in the supernatant fluid of centrifuged urine 0.11—0.53%. The difference is accounted for mainly by bacteria. When the leeches were kept under dry conditions, resulting in 23.30—37.38% loss in body weight, the solid matter rose to 6.75—16.28%

indicating an ability to resorb water in times of desiccation. However, the capacity of surviving arid conditions depended mainly on the amount of blood stored in the crop of these animals.

Estimation of ammonia N and urea N in water containing a leech

The results have been summarized in Table 1. 0.972—1.958 mg of ammonia N and 0.004—0.394 mg of urea N was excreted by each worm within a period of 12—144 hours.

Estimation of ammonia N, urea N, NPN, and total N present in the urine

The results have been summarized in Table 2. On an average the urine contained 35.7 μg ammonia N/ml, 21.6 μg urea N/ml and 484 μg total N/ml. The supernatant fluid of the centrifuged urine contained on an average 44.8 μg ammonia N/ml, 13.3 μg urea N/ml, 126.6 μg NPN/ml, and 177.6 μg total N/ml.

Table 2

Ammonia N, urea N, total N, and NPN, (μg/ml) of the vesicular fluid (urine), collected from the nephridial bladders of Hirudinaria granulosa

Expt.	Urine			Supernatant fluid of the centrifuged urine			
	Ammonia N	Urea N	Total N	Ammonia N	Urea N	Total N	NPN
1	28.3	17.7		55.6	10.5	165.1	138.1
2	48.1	21.1		30.6	13.6	99.9	116.6
3	34.6	12.9		48.4	15.9	103.2	61.6
4	32.1	34.9				143.0	144.2
5						223.1	192.5
6						266.6	228.7
7			546			261.7	185.4
8			318			119.4	82.5
9			480			131.9	85.5
10			897			276.0	45.8
11			180			164.4	111.7
Mean	35.7	21.6	484	44.8	13.3	177.6	126.6

Discussion

From the available literature [14] it appears that studies on the physiology of excretion in leeches have been confined to the uptake of ammonium carminate by the botryoidal tissue and indigo sulphate by the vaso-fibrous tissue of *Hirudo* [8, 15], metabolism of iron in *Hirudo* and *Glossiphonia* [3, 4, 5], phagocytosis by coelomic corpuscles in *Erpobdella* [12], bacteria present in the

nephridial bladders of *Hirudo* [6]. However, HEIDERMANNS [13] analysed the water containing *Hirudo medicinalis* and found that 72% of nitrogen is excreted in the form of ammonia, while amino acids, purines, urea and creatinine each account for 5—10%. Although keeping the leeches in water is a convenient method for obtaining their excretory fluid, the water is bound to contain mucoproteins, secreted by the slime glands present in the skin, faeces and the substances discharged through the mouth in addition to the nephridial fluid. HEIDERMANNS [13], therefore, tested not only the nephridial fluid but also the mucus and the discharges from the two ends of the gut. The author has, for the first time, analysed pure leech-urine, collected directly from the nephridia. About 32% of the total NPN is excreted by the leeches in the form of ammonia N, and 14% as urea N. Rest of the NPN is excreted mostly in the form of unchanged amino acids [9, 10, 11] — a significant feature of certain invertebrates [1]. The appreciable leakage of the amino acids and proteins in the animals presumably promotes the bacterial growth in the nephridial bladders to the extent of about 8—69% of the total N of the urine [11].

Results of the analysis of water containing leeches are interesting. The amount of ammonia N and urea N present in the water was not always proportional to the duration of the experiment. This may either reflect "flushing out" of nephridia due to immersion in distilled water, or may be due to decomposition of urea and evaporation of ammonia.

Average size of the terminal vesicles of the pre-testicular nephridia is significantly smaller as compared to those of the testicular nephridia.

Acknowledgment

The author is grateful to Prof. G. H. BOURNE, Anatomy Department, Emory University, Atlanta, U.S.A., and to Dr. K. H. MANN, Department of Zoology, University of Reading, England, for giving advices and correcting the manuscript of the paper; to Dr. (Miss) USHA GUPTA and Prof. S. P. GUPTA, Department of Pathology and Bacteriology, King George's Medical College, University of Lucknow, and to Prof. D. G. EVANS, London School of Hygiene and Tropical Medicine, England, for constant help and encouragement.

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UNTERSUCHUNGEN ÜBER DIE EXKRETION DES INDISCHEN EGELS *HIRUDINARIA GRANULOSA* (SAVIGNY)

Mit Hilfe einer eigenen Methode untersuchte Autor als Erster reinen, direkt von den Nephridien gewonnenen Egel-Harn. Mit Conway's Mikrodiffusions Technik wurde der Stickstoff-Gehalt des Harns und des Supernatans von zentrifugiertem Harn entsprechend folgenden Fraktionen: Ammonia-N, Harnstoff-N, Nicht-Protein-N (NPN), Total-N quantitativ bestimmt. Kleinere Modifikationen, so z. B. die Conway-Einheiten betreffend werden vorgeschlagen. Über 32% des NPN wird in Form von Ammonia, und 19% als Harnstoff-N ausgeschieden. Der Rest wird hauptsächlich mit freien Aminosäuren ausgeschieden. Es wurden im Weiteren Größe und Form der Nephridialen-Blasen, die Menge des gebildeten Harns, seine physikalischen Eigenschaften und das mikroskopische Bild, sein pH und der Wassergehalt untersucht. Zum Vergleich wurde auch der Ammonia-N und Harnstoff-N Gehalt des Wassers, in dem die Egel gehalten wurden, quantitativ bestimmt. Schliesslich wurde auch der Einfluß des Haltens unter trockenen Bedingungen auf den Wassergehalt untersucht.

ИССЛЕДОВАНИЕ ВЫДЕЛЕНИЙ У ПИАВОК *HIRUDINARIA GRANULOSA* (SAVIGNY)

При помощи новой разработанной автором техники впервые проводится анализ чистой мочи пиавок *(Hirudinaria granulosa)*, взятой непосредственно из почек. Количественное определение азота в аммонии, азомочевины, неазопротеина и общего азота в моче и в надосадочной жидкости центрифугированной мочи проводилось при помощи техники микродиффузии по Конуэию. Некоторые изменения были предложены для очистки единиц Конуэя. Около 32% общего неазопротеина выделяется в виде азота в аммонии и 14% — в виде азомочевины. Остальная часть выделяется преимущественно в виде свободных аминокислот. Исследовались также размеры и внешний вид почечных пузырьков, общее количество чистой моче, физикальные свойства, микроскопические признаки, pH мочи и содержание воды в моче. В целях сопоставления проводилась количественная оценка азота в аммонии и азомочевины в воде, содержащей пиавки. Кроме того исследовалось действие жажды на содержание воды в моче животных.

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Acta Biol. Hung., **15**(4), 431—457 (1965)

ELECTRON MICROSCOPY OF THE MEDIAN EMINENCE OF THE RAT

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(Received September 20, 1964)

Synopsis

The fine structure of the median eminence in the rat has been examined. In the cells of the ependymal layer moderatelly developed Golgi apparatus, mitochondria, ergastoplasm and free ribosomes as well as many lipid droplets can be found. Similar droplets can also be detected in the processes of the ependymal cells extending to the cerebral surface and in the ependymal feet. The layer under the ependyma is rich in glial cells. In certain of these cells, dense bodies similar in size to mitochondria can be found resembling Gomori-positive granules visible in these cells by light microscopy. The fibrous layer is composed of thick (0.5 to 1 μ) and thin (0.08 to 0.5 μ) nerve fibers. The outer zone (palisade zone) is made up of axon endings, containing two types of vesicles. The smaller, electron-translucent vesicles, 240 to 400 Å in diameter, are similar to synaptic vesicles, whereas the bigger vesicles (450 to 1,250 Å) have a dense content (dense-core vesicles). The latter resemble both the elementary neurosecretory granules and the cathecolamine granules. The capillaries of the portal vessels are fenestrated. The pores of the endothelial cells are facing the axon endings of the palisade zone. In the area between the surface of the median eminence and the pars tuberalis, unmyelinated nerve fibres can be found, which presumably are vasomotor fibres coming from the carotid plexus. The nature of the nerve fibres in the median eminence and the release mechanism of the secretory substance is discussed in the text.

Introduction

The median eminence is an area of the hypothalamus playing an important role in the control of the anterior pituitary [8, 9]. According to histological and physiological investigations, informative substances formed in certain hypothalamic cells are transmitted in this area to the portal vessels of the pituitary. The fine structural details of this region may greatly contribute to the understanding of the mechanism of transport of these substances. The electron microscopic structure of the median eminence has been examined by BARRY and COTTE [3] in the guinea pig, KOBAYASHI and co-workers [11] in the parakeet, OOTA and KOBAYASHI [23, 24] in the pigeon and frog, OOTA [21, 22] in the mouse and turtle, and BRADBURY and HARRIS [4] in the rabbit. Each author has described a number of unmyelinated nerve fibres as well as glial and ependymal feet situated around the portal vessels. Some of the axon endings contain synaptic vesicles, while others neurosecretory vesicles or both.

The structure of the median eminence is not identical in the various species. There are differences in size and quantity of the vesicles and in the ratio of nerve endings and glial and ependymal feet. In certain species, the capillaries of the area concerned are fenestrated.

Since the majority of physiological and experimental morphological examinations on the hypothalamic-hypophyseal system have been made on rats, the present paper is intended to supply further data as to the fine structure of the median eminence of rats. The paper by KOBAYASHI and co-workers [12] on the same subject was published at a time the observations described here were made. The results we obtained mostly support KOBAYASHI's observations and in part offer supplementary data.

Material and method

Adult albino rats kept under normal laboratory conditions were used.

After decapitating the animal, the base of the cerebrum was exposed and fixative was dropped on the hypothalamic region. An interval of 40 seconds passed from the decapitation of the animal to the applying of the fixative. Two minutes later, a specimen $2 \times 2 \times 2$ mm in size was cut out containing the median eminence which was postfixed in 1 per cent phosphate-buffered OsO_4 solution [16] for 2 hours and then embedded in Vestopal W. The sections cut with a Porter-Blum ultramicrotome were stained with plumbite ions according to the method suggested by MILLONIG [17], and examined in a JEM 6c electron microscope.

Results

Light microscopy

The ventricular surface of the median eminence is composed of a series of ependymal cells under which a wide fibrous layer is situated. The upper zone of the fibrous layer is rich in cells and myelinated nerve fibres ("hypendyma"), while in the lower part, cross sections of fibres containing neurosecretory substance can be seen (tr. hypothalamo-hypophysealis). The outer zone (palisade zone) is striated because of the ependymal and glial processes running at right angles to the surface of the brain and the axon endings leaning out to the surface. The contact layer located between the median eminence and the pars tuberalis is made up of flattened connective tissue cells (pia mater) and a number of capillaries and arterioles ("Mantelplexus"). The latter emit capillary loops into the inner parts of the neural tissue of the median eminence (portal capillaries, "Spezialgefäße").

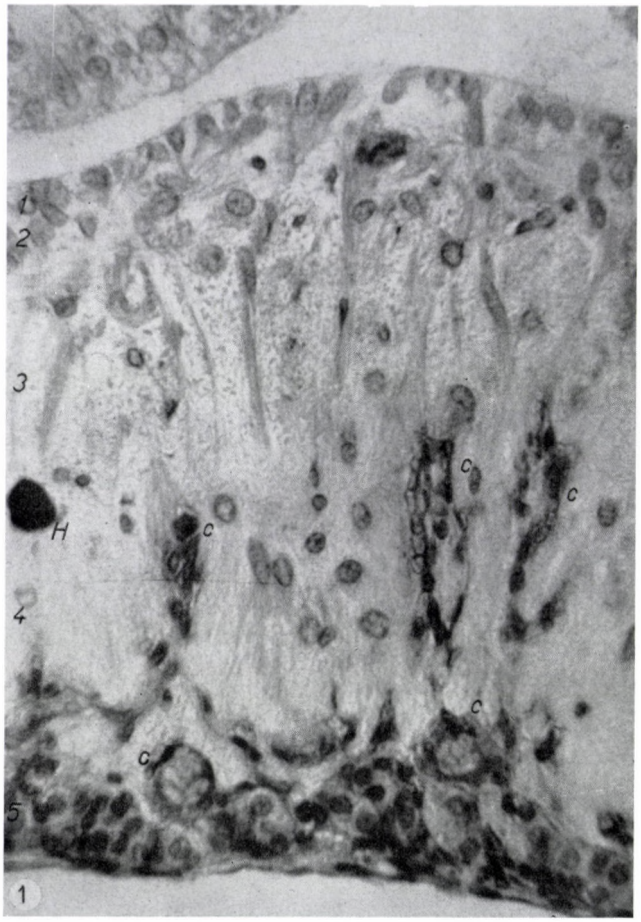

Fig. 1. Frontal section of the median eminence of the rat. Light microscopic specimen stained whith the chrome-haematoxylin phloxine method. 1: ependymal layer, 2: hypendyma, 3: fibrous layer, 4: palisade zone, 5: pars tuberalis, c: primary plexus and portal capillary loops H: Herring-body

Electron microscopy

Ependyma

The form and arrangement of the ependymal cells is rather irregular (Figs 2, 3). The base of the cells is extended in long processes piercing the texture of the median eminence and ending on the surface in the form of ependymal feet. The cells contain besides the nucleus a moderately developed Golgi apparatus, mitochondria, ergastoplasmic profiles, free ribosomes and a varying quantity of 70 to 100 Å thick filaments often arranged in bundles. The apical parts of the cells are bound together by means of desmosomes. The structure of the ependymal cells corresponds in its main features to that described by

BRIGHTMAN and PALAY [6]; the only difference lies in the fact that cilia cannot be observed on the cell surface. Instead, only a few irregular microvilli can be found. In this area the ependymal cells have been found to contain a larger number of lipid droplets [30, 18]. Similar lipid droplets could also be detected in the ependymal processes and feet.

Hypendyma

The structure of glial cells found here resembles that of the ependymal cells. Besides some fine filaments, mitochondria and ergastoplasmic profiles, a well-developed Golgi apparatus and free ribosomes can be seen in the cytoplasm (Fig. 3). Dense bodies of mitochondrial size can often be observed (Fig. 4). Their irregular protuberant outlines and inner structure suggest that they are composed of minor globular units. Their substance consists of fine granular material of varying density. In places a limiting membrane can be detected on them. The size and structure of the bodies indicates a striking similarity to the lipofuscin granules as described in electron microscopy. According to their size and location, they may be regarded to be identical with the Gomori-positive granules observed in these cells by light microscopy.

In the same area, typical large astrocytic processes with watery cytoplasm and glial filaments running in bundles can be observed. They form a layer consisting of characteristic glial feet (membrana limitans gliae) around the vessels.

The majority of nerve fibres in this layer are unmyelinated; certain fibres, however, possess a thin myelin sheath (Fig. 5). The myelinated fibres vary in diameter from 1 μ to 4 μ, but apart from some mitochondria and neurofilaments in the axoplasm, we have failed to detect granules of neurosecretory substance in them. The diameter of the unmyelinated axons is extremely variable; in some of them, synaptic vesicles are present, while in others light and dense vesicles, similar to the neurosecretory elementary vesicles, were to be seen (Fig. 5). In certain fibres there was a strikingly large number of mitochondria, some of which showed signs of degeneration.

The fibrous layer

This layer is divided into perpendicular columns by the ependymal processes running from the cerebral ventricle towards the surface of the median eminence. In the processes, filaments (Fig. 6), elongated mitochondria and chains of the endoplasmatic reticulum are running parallel with the direction of their downward course. In some places groups of lipid droplets can be seen.

The regions lying between the ependymal processes are filled with numerous unmyelinated fibres. In a minor part of them, dense-core vesicles with a diameter of 1,000—1,200 Å can be found which resemble the elementary

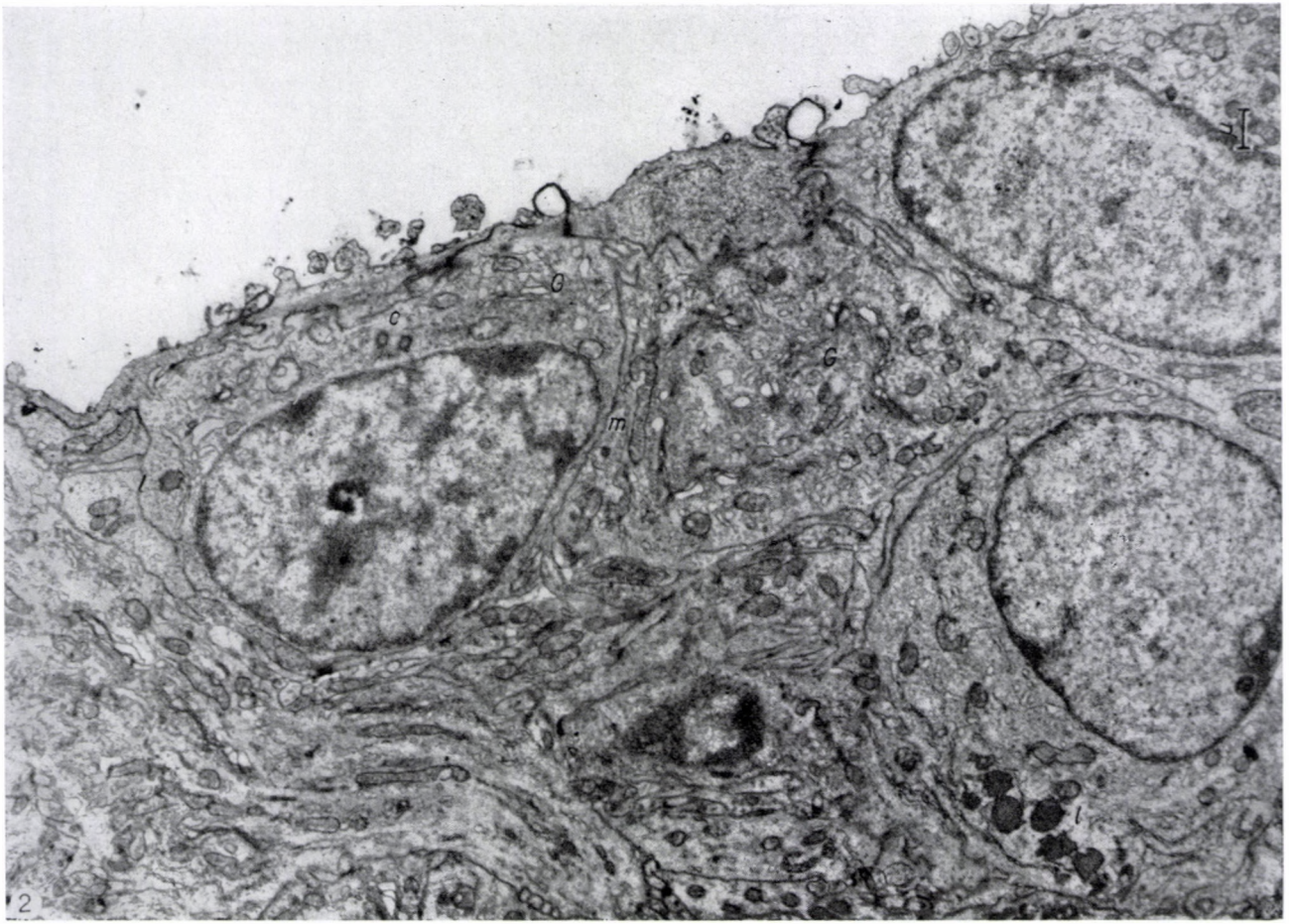

Fig. 2. Detail of the ependymal layer. The ependymal cells form a rather irregular layer. Centrioles (c), Golgi-apparatus (G), mitochondria (m), lipid droplets (l) and free ribosomes can be observed in the cytoplasm

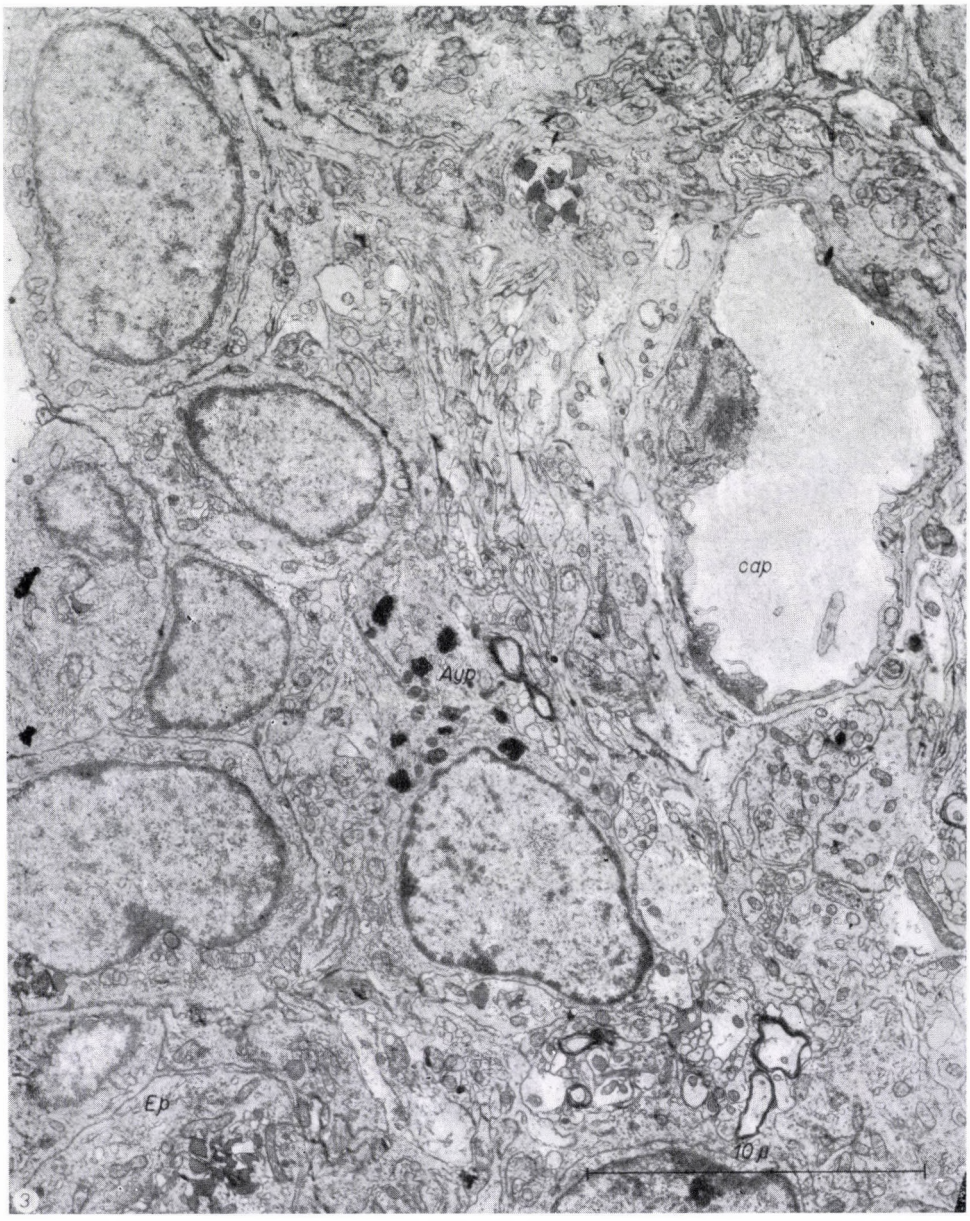

Fig. 3. A general view of the ependyma and hypendyma, the first two layers of the median eminence. The irregular arrangement of the cells of the ependymal layer (ep) and a hypendymal glial cell (hyp) can well be observed. Note the continuity of the endothelium (no fenestration) on the capillary (cap)

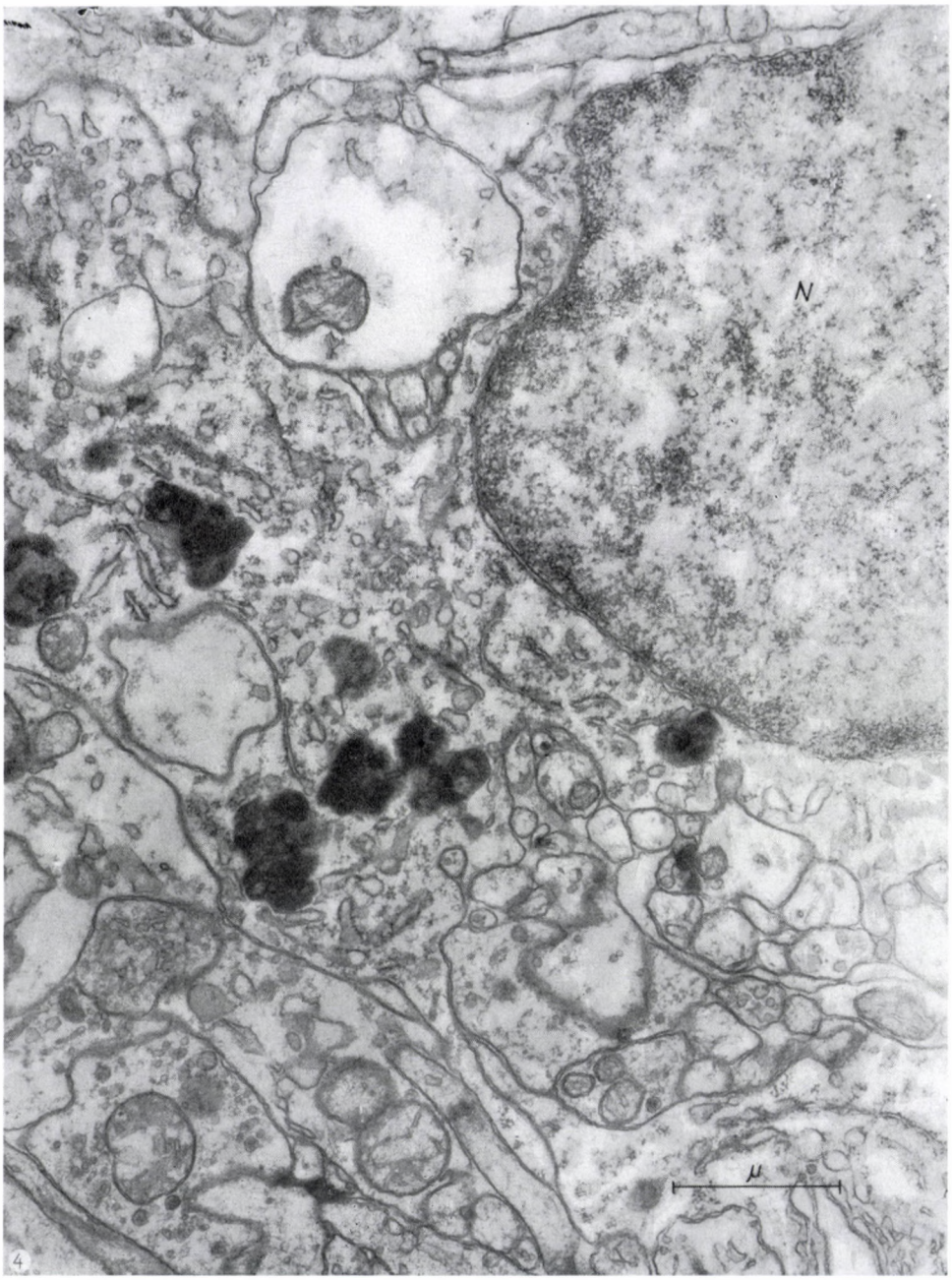

Fig. 4. Hypendymal cell. Many tubules of the endoplasmic reticulum are seen to the majority of which ribosomes are attached. In addition, free ribosomes and dense bodies of a size similar to that of mitochondria can be observed. The substance of the bodies is composed of minor, globular, dense areas and is finely granulated. N nucleus

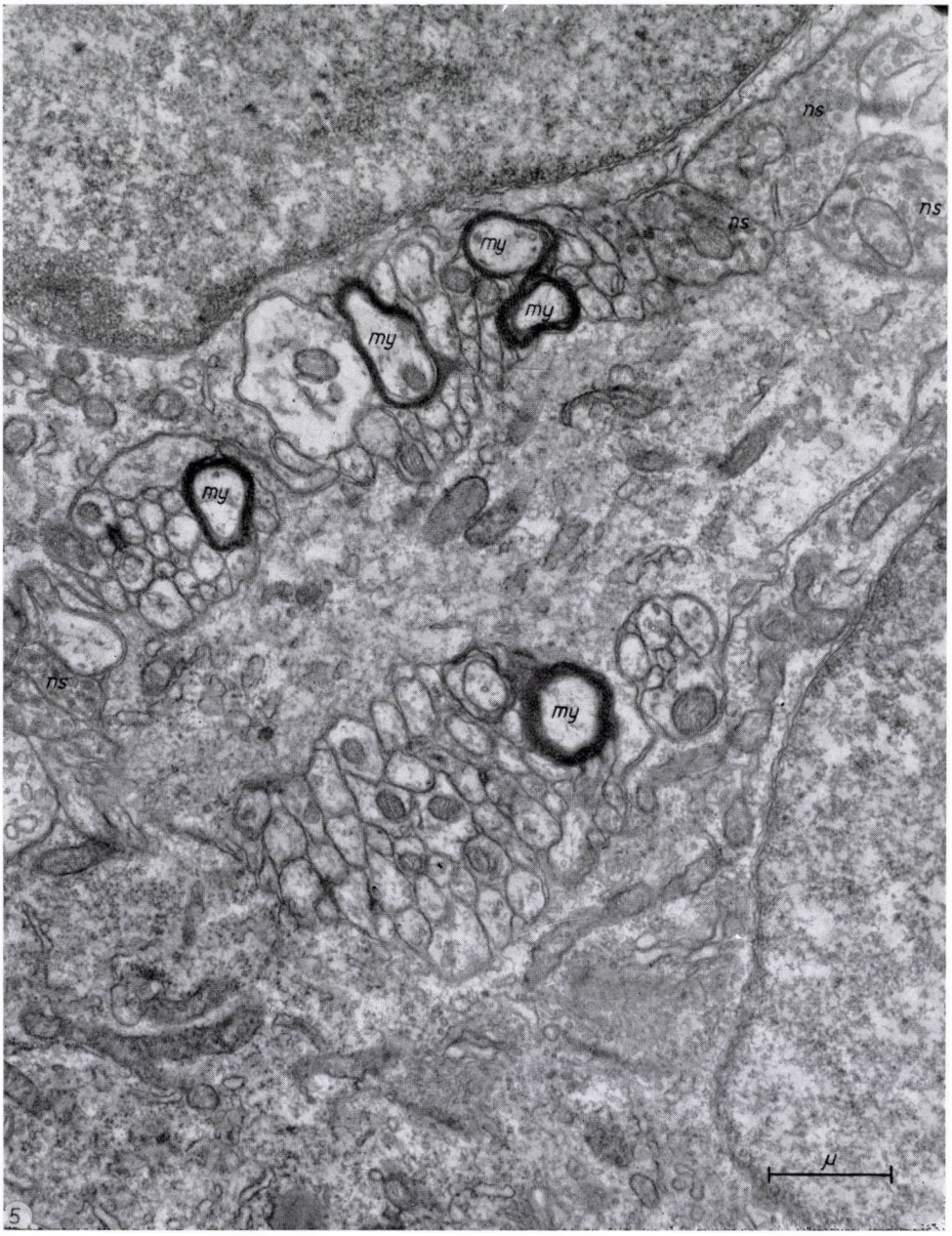

Fig. 5. Detail of the hypendyma. Between two hypendymal glial cells, cross sections of nerve fibres can be seen. Some of the fibres have a thin myelin sheath (my), while in others neurosecretory vesicles can be found (ns)

neurosecretory vesicles. Empty vesicles of the same diameter may join them in their axons. In the majority of cases, axons most frequently containing dense-core ("neurosecretory") vesicles are large in diameter (0.5 to 1 μ), (fibres of the hypothalamo-hypophyseal tract?). Most of the fibres are extremely thin, with a mean diameter of 0.3 μ (between 0.08 to 0.5 μ). Neurotubules of about 200 Å in diameter are often observed in the fibres. They contain mitochondria and dense-core vesicles. The frontal section of the median eminence most frequently shows the cross-sections of the fibres; at many sites, however, it is clearly discernible that the thin fibres turn downwards parallel with the ependymal processes (Fig. 6.). In these fibers, empty and dense-core vesicles alike can be found in larger quantities. Occasionally, synaptic vesicles also occur in the fibres.

Palisade zone

The lowest, superficial layer of the median eminence is the palisade zone (Fig. 7). In contrast to other regions of the central nervous system, here the surface is made up of ten thousands of axon terminals instead of a coherent layer of glial and ependymal feet (membrana limitans gliae externa).

The nerve terminals are irregular, bulbous swellings of the axons. They vary greatly in shape and size. Most of them vary between 0.5 and 1 μ, but sometimes they are as large as 2.5 μ. The free surface of the terminals takes part in the formation of that of the median eminence. The cytoplasm of the axon endings is of a fairly compact structure as a result of the presence of the numerous vesicles it contains. On the basis of their size, the vesicles can be divided into two main groups: 1. small, seemingly empty vesicles, 240 to 400 Å in diameter and 2. vesicles from 450 to 1,250 Å in diameter (Fig. 8), most frequently with a dense content.

There is a striking similarity between the former small, electron-translucent vesicles and the so-called synaptic vesicles which are considered characteristic for the majority of synapses. Vesicles of this type can be found in almost every axon ending of the median eminence, irrespective of whether or not it contains the other type of vesicle. In some cases these vesicles are densely packed and form smaller or larger groups, some of which are situated under the plasma membrane facing the pial surface and appear to be closely related to it (Figs 9 and 10).

The average size of the larger vesicles (from 450 to 1,250 Å) is 770 Å. In the interior of most of them a dense content can be observed by which they resemble the "elementary neurosecretory granules" [1, 2]. They differ, however, from the neurosecretory vesicles in some respects: their content is not so compact and homogenous, it fails to fill the vesicle completely, and the vesicles are somewhat smaller than those to be found in the neurohypophysis.

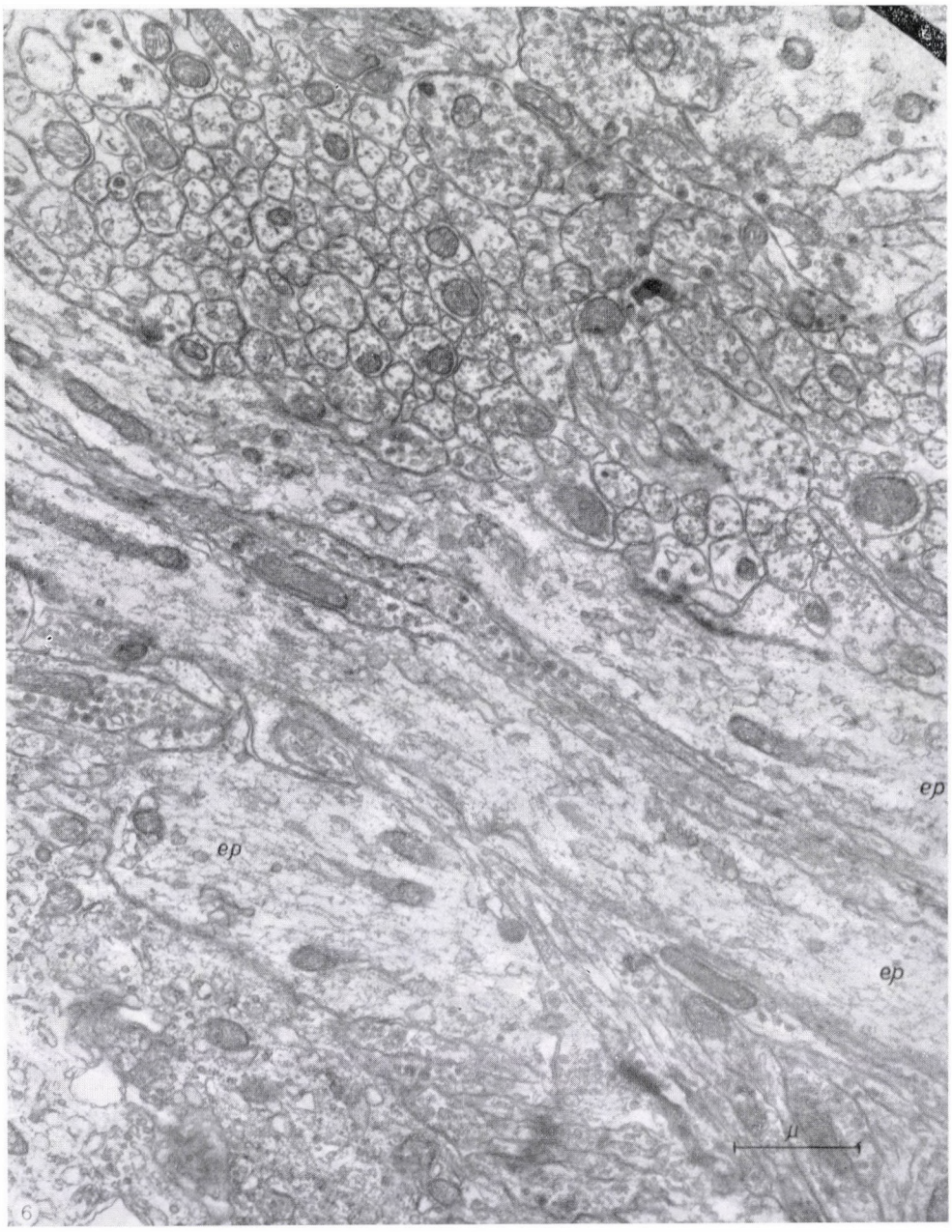

Fig. 6. Detail of the fibrous layer. The mass of the nerve fibres is divided by thick ependymal processes (ep) into columns. In the thin nerve fibres vesicles of neurosecretory or synaptic character can be observed

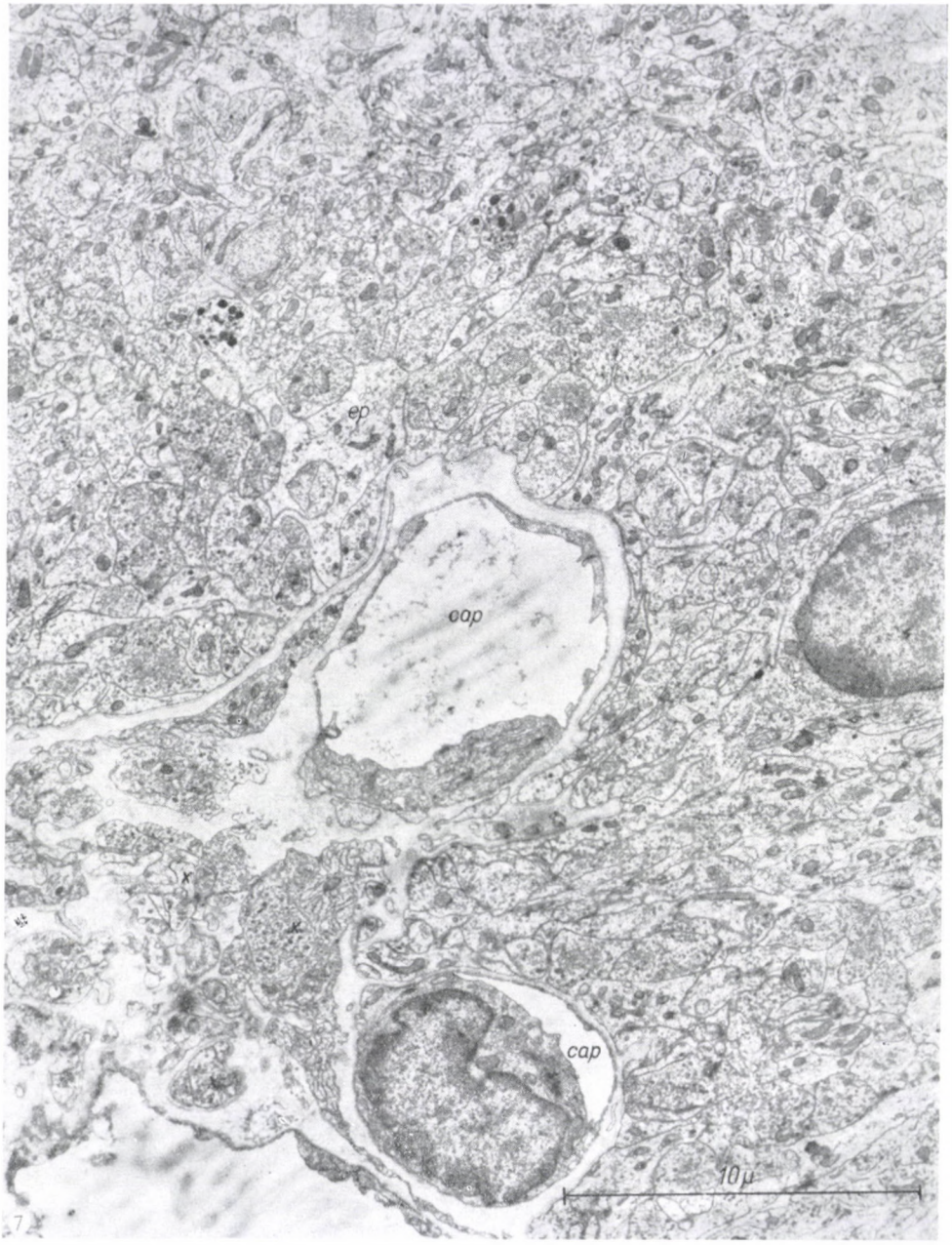

Fig. 7. General view of the palisade zone. The surface of the median eminence is highly folded by the capillaries of the portal vessels. This layer is composed of numerous axon endings containing vesicles and of a smaller number of glial and ependymal feet. The structures (x) under the surface of the median eminence are presumably tangential sections of the palisade zone

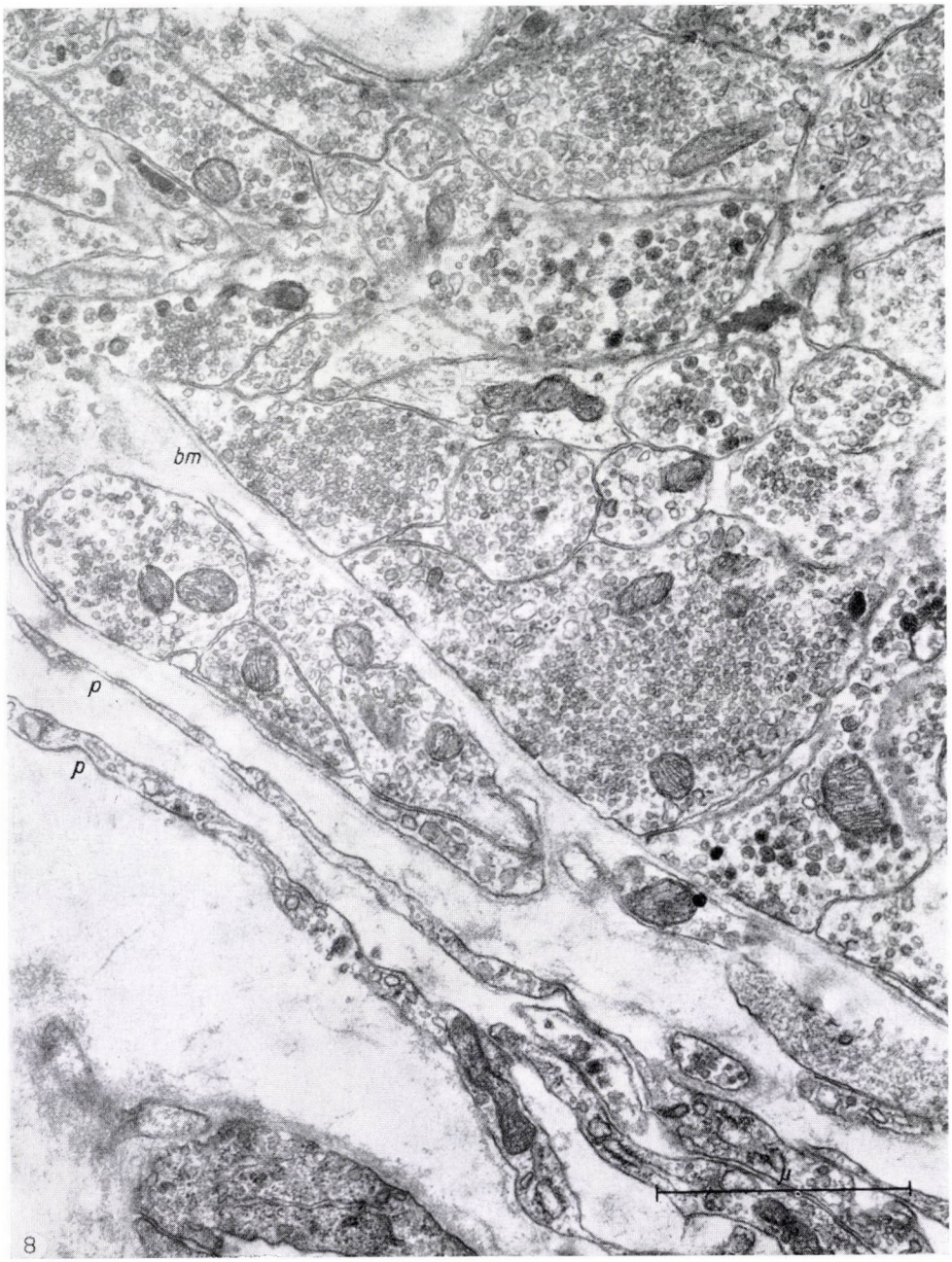

Fig. 8. In the axon terminal of the palisade zone there are two types of vesicles to be found. One of them is a small electron translucent ("synaptic") vesicle, while the other one is larger and has a dense content. bm: basement membrane, p: pial cells, cap: capillary

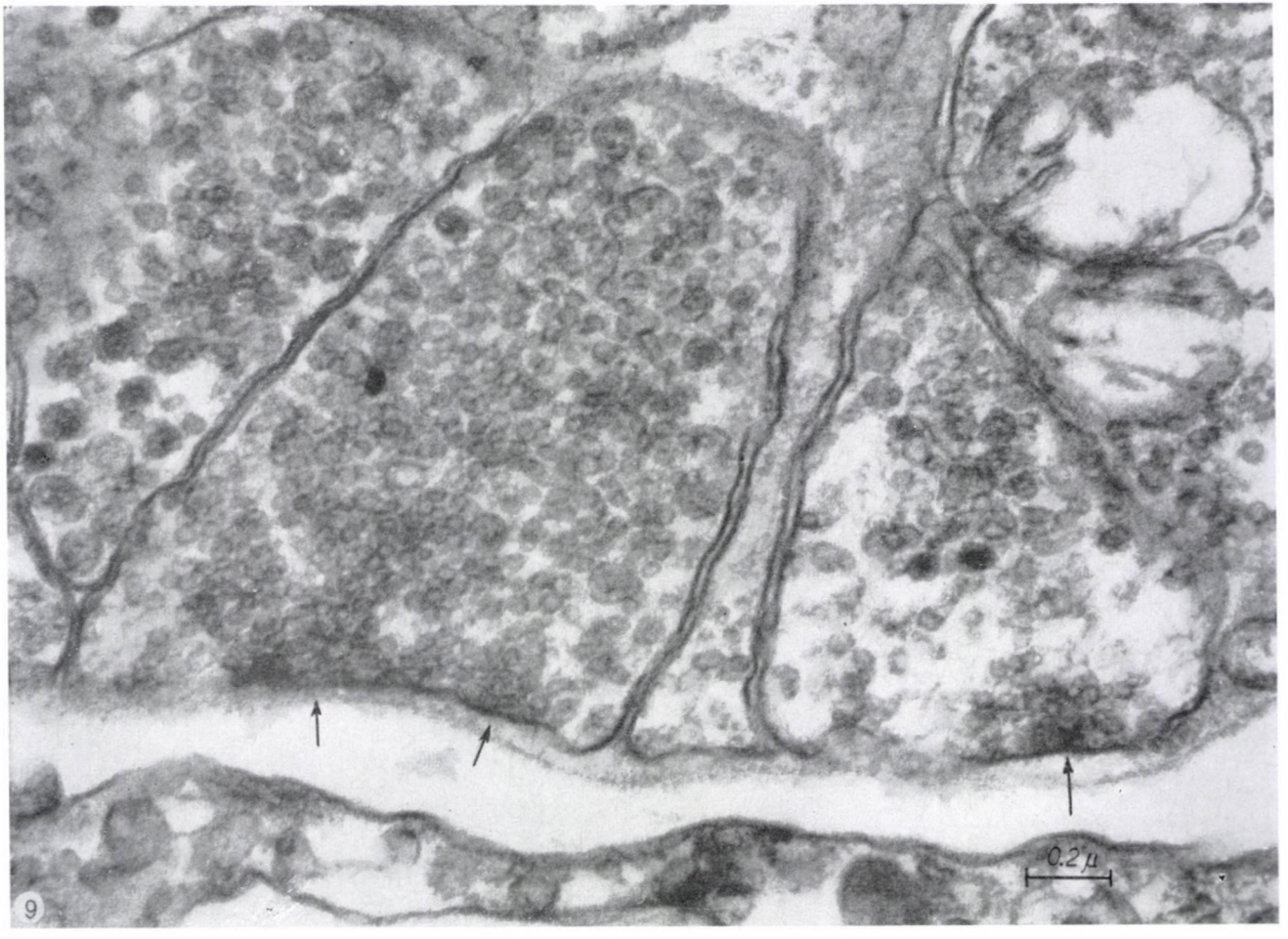

Fig. 9. Axon endings in the palisade zone. The "synaptic" vesicles frequently form groups under the cell membrane (arrow)

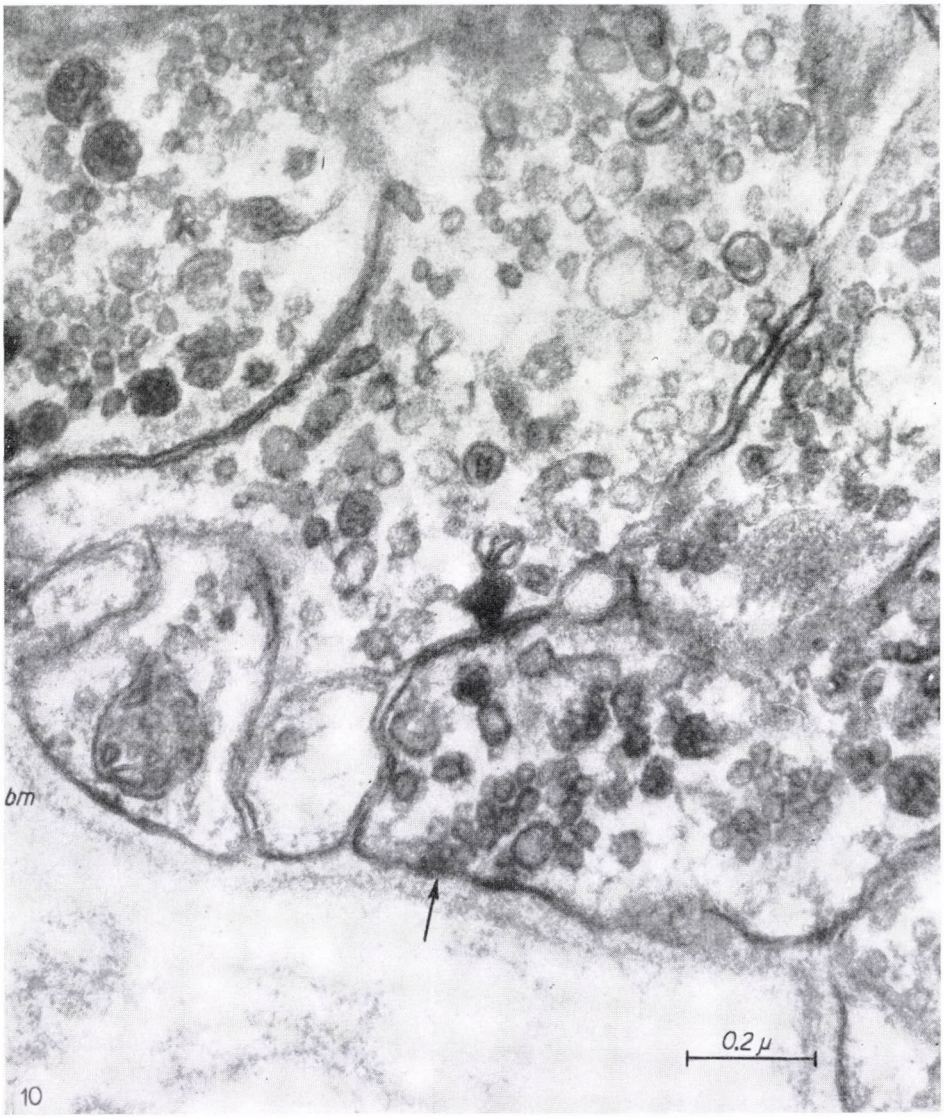

Fig. 10. Axon endings in the palisade zone. "Synaptic" and dense-core vesicles are equally seen. Note the grouping of the small ("synaptic") vesicles under the cell membrane (arrow). bm: basement membrane

In most cases the cristae of the mitochondria in the axon endings are oriented longitudinally. In some endings circular, dense bodies may occur, which are most likely to correspond to degenerating mitochondria.

Among the nerve terminals, ependymal and glial feet are interposed in which lipid droplets, mitochondria, fine filaments and profiles of the endo-

plasmic reticulum can be seen. In the median line, axon endings can be found in greatest number, with only a few ependymal and glial feet among them. Dense, spherical or elongated bodies of varying size with a fine granulated or homogenous substance can frequently be observed in them (Fig. 11). Laterally, the number of ependymal and glial feet gradually increases to constitute a conntinuous membrana limitans gliae. The cytoplasm of the feet in the lateral regions of the median eminence is markedly empty, apart from some concentrically lamellated bodies.

Capillaries

The largest number of capillaries can be found on the contact surface between the median eminence and the pars tuberalis. The capillaries of the primary plexus ("Mantelplexus"), and the portal capillary loops ("Spezialgefäße") lying in the deep folds of the cerebral surface show a characteristic structure. That side of the capillary endothelium which is facing the axon endings of the palisade zone is extremely flattened (350—400 Å thick), and it is pronouncedly fenestrated (Figs 7, 8 and 12). The pores are about 700 Å in diameter, with a thin diaphragm which appears to bridge the gap. The nucleus and cell organelles are always to be found on the opposite side of the capillary, embedded in a wider cytoplasmic layer of the endothelium (Fig. 12). The axon endings are separated from the vessel lumen by the following layers: the basement membrane of the cerebral surface (450—500 Å), a space varying in thickness and often containing connective tissue fibrils or processes of connective tissue cells in it, the basement membrane of the capillary and the fenestrated endothelium.

The common occurrence of axon endings and fenestrated endothelium is particularly demonstrable if areas in which the axon endings are missing are examined. In these places (the frontal region of the median eminence (Fig. 13), the subependymal vessels (Fig. 3) and the lateral parts of the median eminence (Fig. 14)), the typical picture of the cerebral capillaries or that of the capillaries in general can be observed.

Contact surface

The surface of the median eminence is separated from the pars tuberalis by capillaries or arterioles of the Mantelplexus, flat connective tissue cells (cells most likely to belong to the pia), and a few connective tissue fibrils (Figs. 8, 12, 14 and 15.).

A problem not yet cleared by light microscopic observations is: whether or not neural structures can be found in this space, and if so, what is their nature like? Two kinds of neural structures could be demonstrated in our observations. One type (Figs 7, 8 and 16) is composed of several nerve ele-

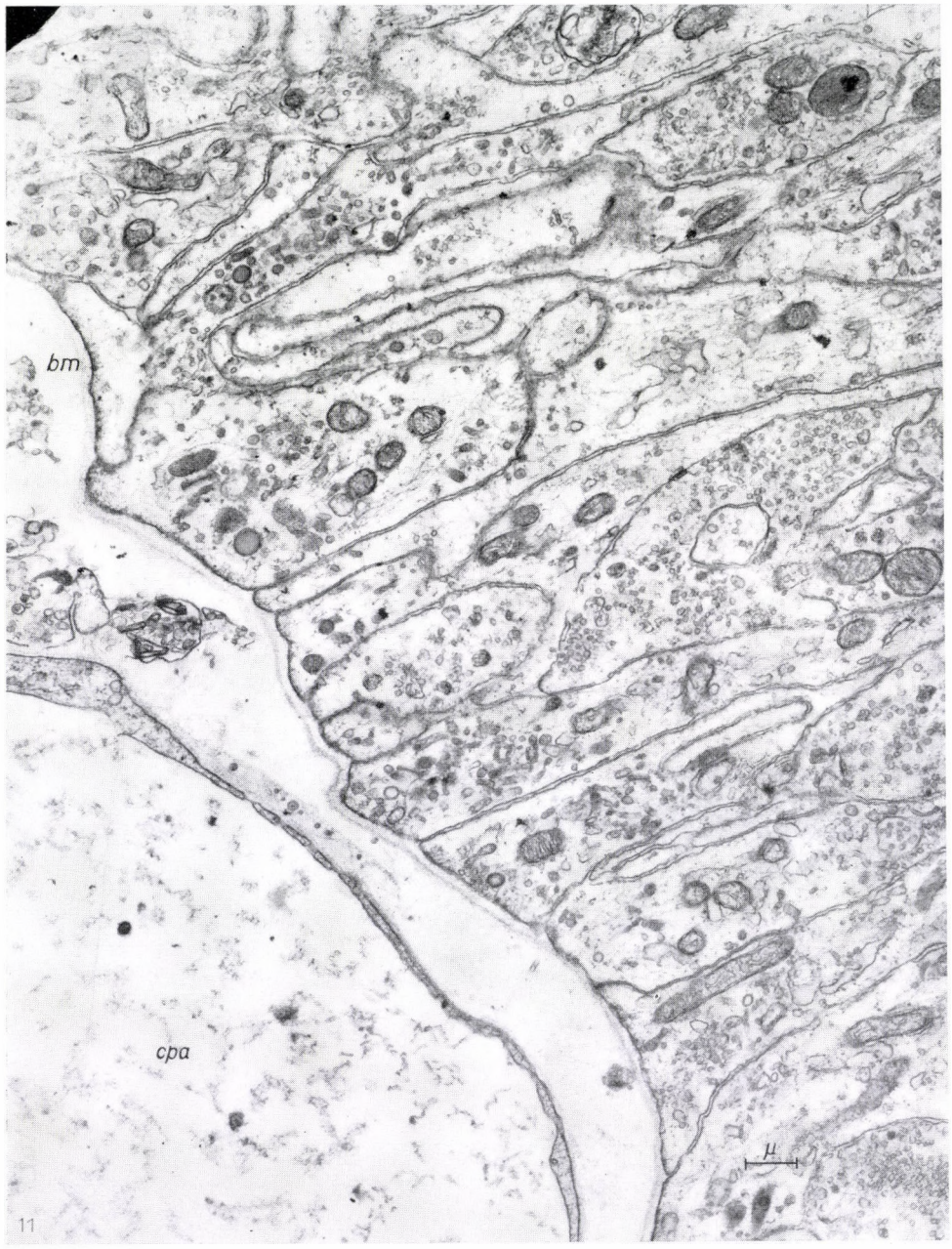

Fig. 11. Ependymal and glial feet of the outer zone in the lateral part of the median eminence. The feet contain fine filaments, few mitochondria and in several places dense globular or elongated bodies (db) of varying size. The endothelium of the capillary (cap) shows only several pores. bm: basement membrane

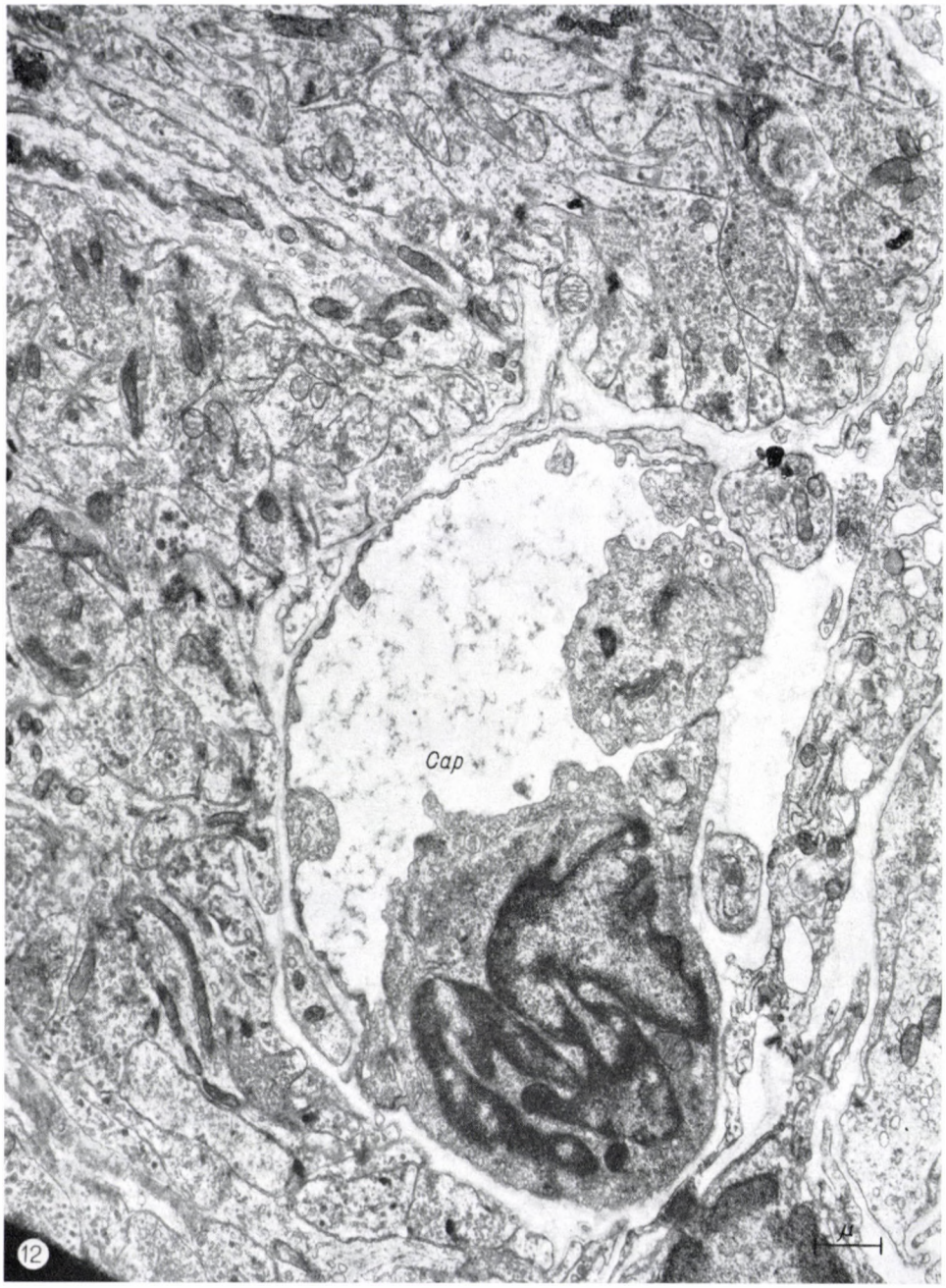

Fig. 12. Capillary of the primary plexus. The endothelium is extremely flattened and fenestrated on the side facing the nerve terminals. A considerable mass of the cytoplasm and the nucleus of the endothelium can be found on the opposite side

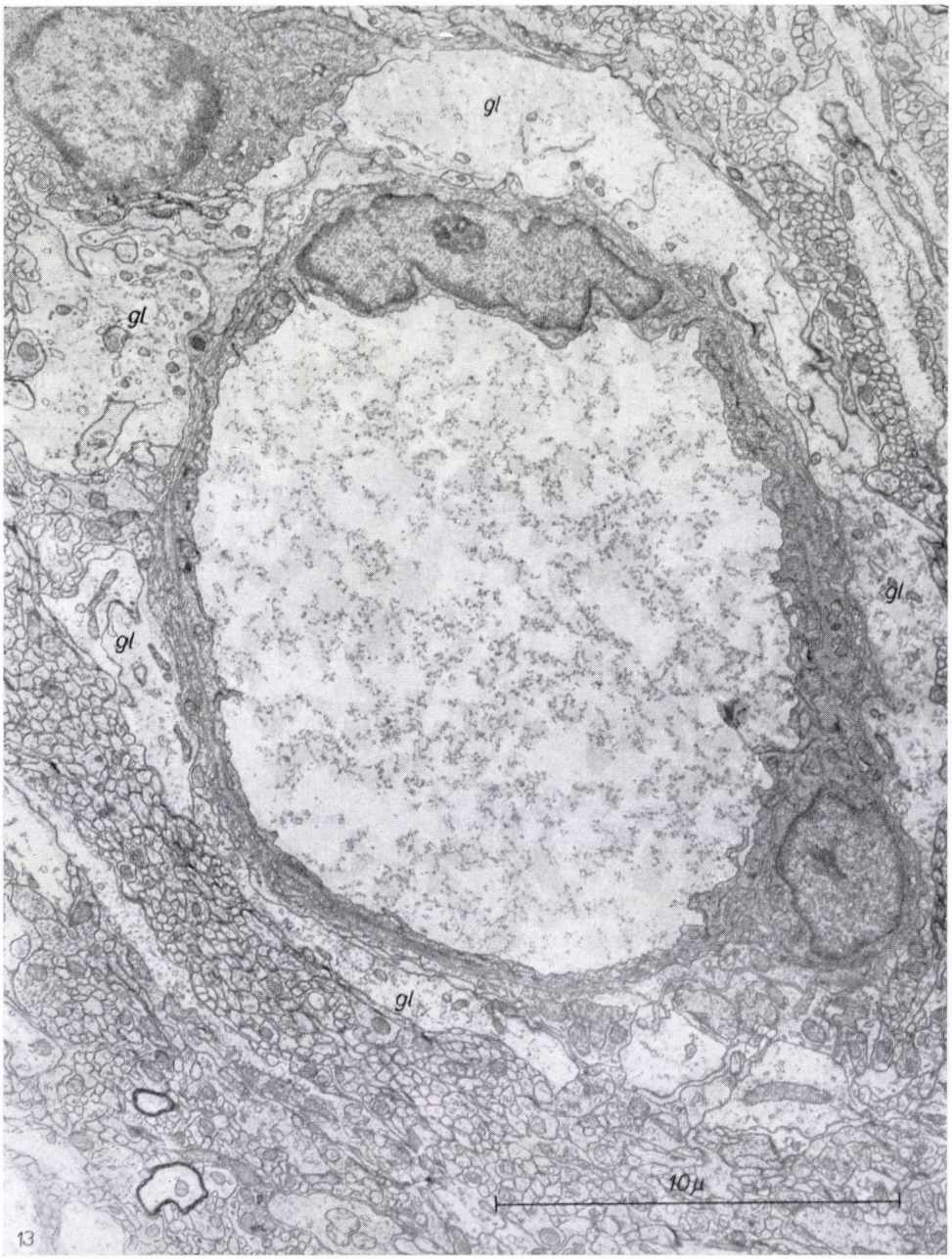

Fig. 13. Cross-section of a vessel in the rostral region of the median eminence. It shows the well-known structure of the cerebral vessels: the non-fenestrated endothelium is surrounded by a basement membrane and glial (gl) feet

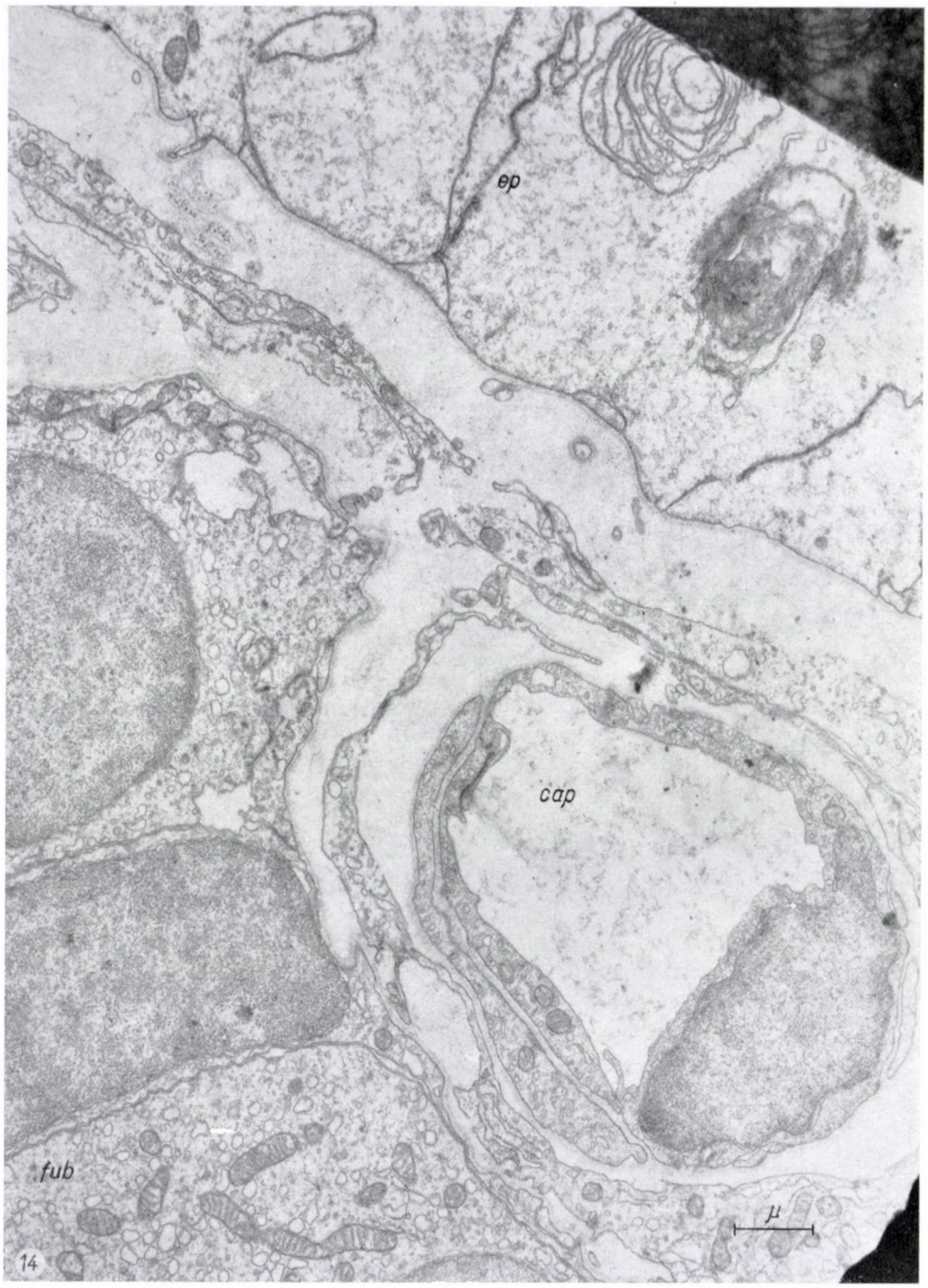

Fig. 14. Capillary from the lateral part of the median eminence. Note that the endothelium of the capillary is not fenestrated. The outer zone of the median eminence consists here of ependymal (ep) feet of empty cytoplasmic structure

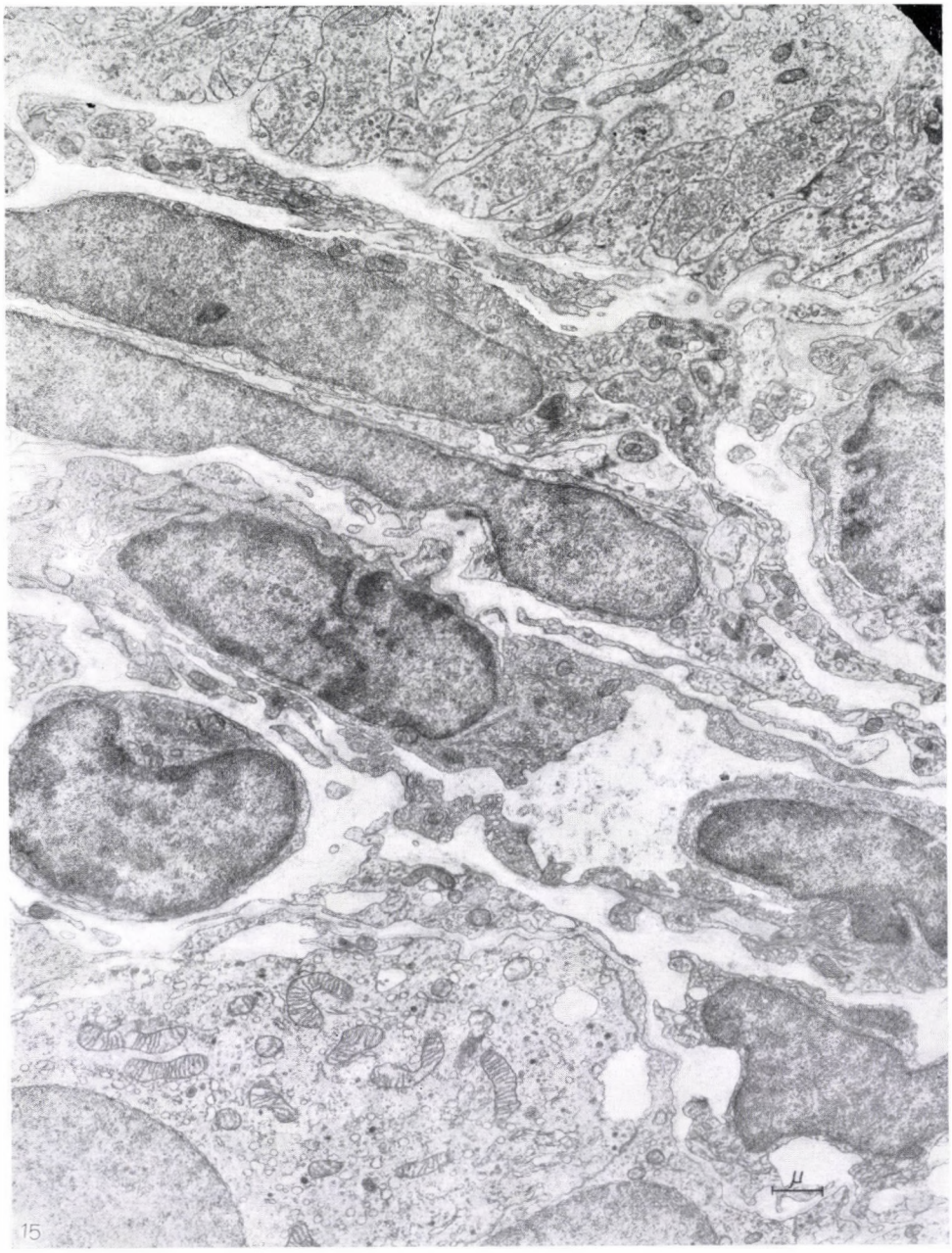

Fig. 15. Contact surface between the pars tuberalis and the median eminence. This layer is composed of vessels and flattened cells of the pia mater

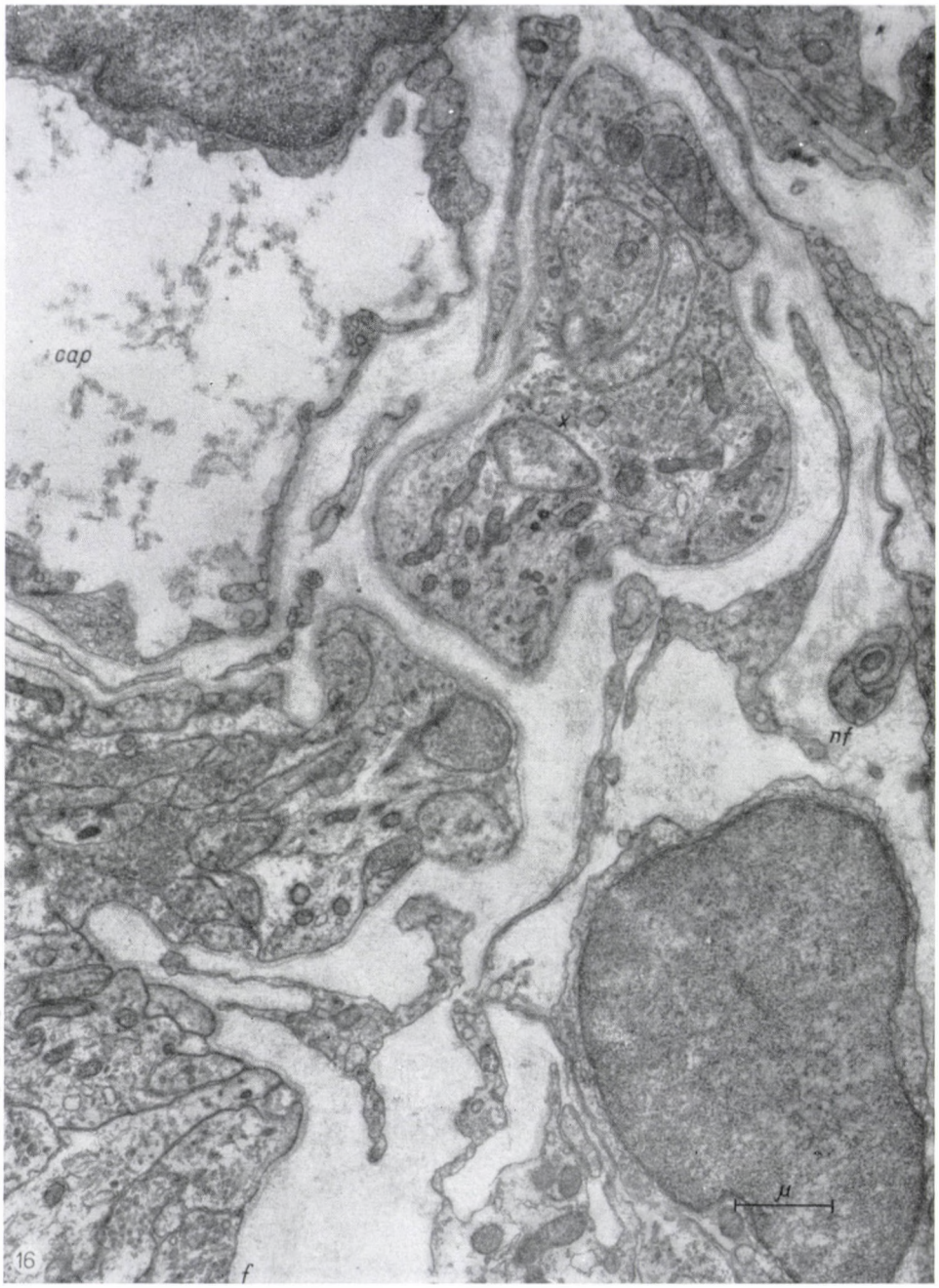

Fig. 16. The contact surface is filled by vessels (cap), tangential sections (x) of the protuberant surface of the median eminence, connective tissue cells and fibrils (f). An unmyelinated nerve fibre (nf) can also be seen

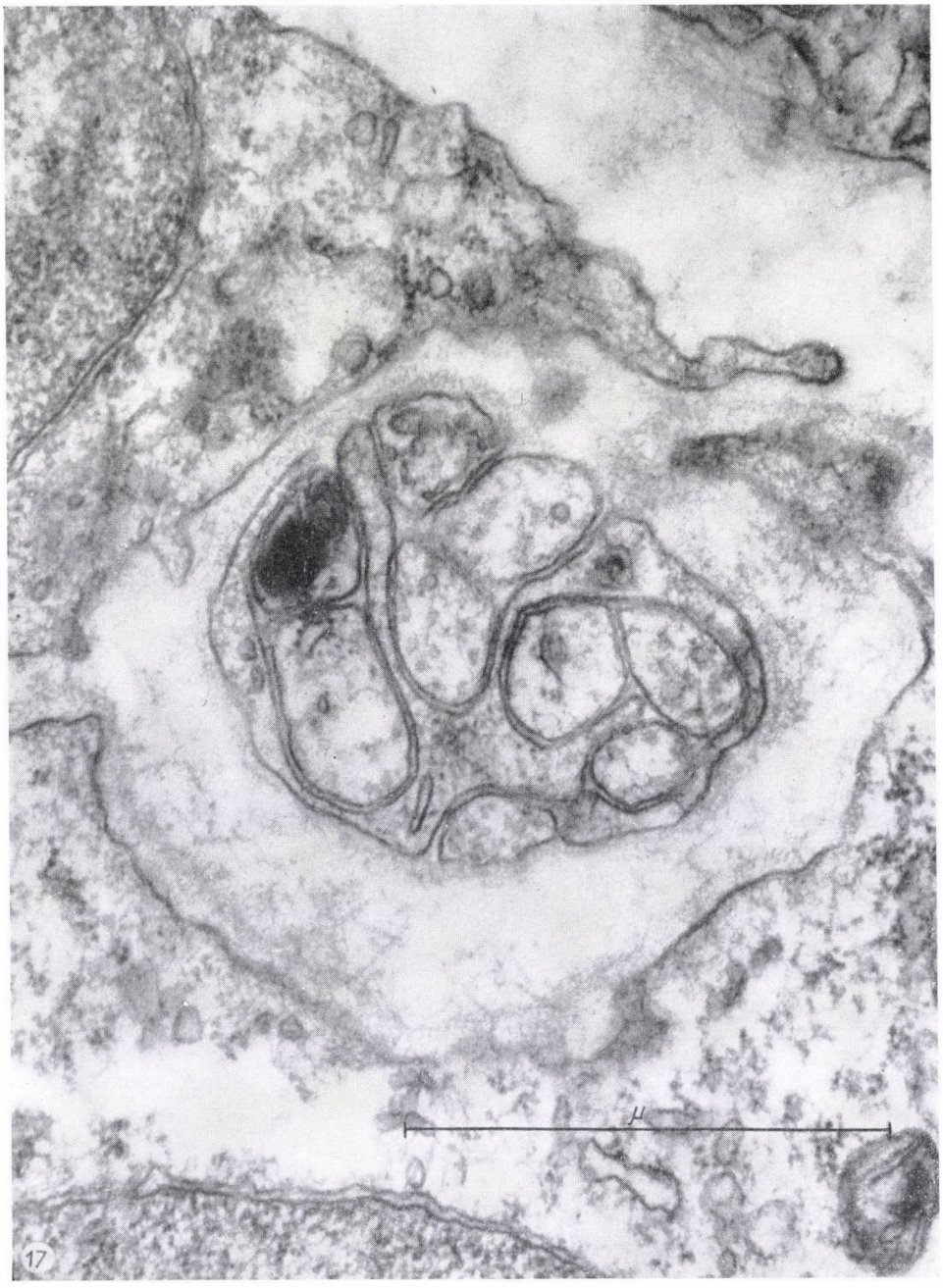

Fig. 17. Cross-section of a peripheral unmyelinated nerve fibre on the surface of the pars tuberalis

ments, without any sheath cell attached, containing vesicles characteristic for the axon terminals of the palisade zone. The whole pattern is surrounded by a basement membrane. It is highly probable that it corresponds to the tangential section of the protuberances of the palisade zone which has an extremely irregular surface. We have been unable to observe fibres of such character penetrating the pars tuberalis. In some cases, we have detected cross-sections of nerve fibres in the connective tissue cells (without mesaxon !). Such structures are likely to correspond to the nerve terminal stretching out of the surface of the median eminence and pressing in the connective tissue cell.

The other neural structure is represented by characteristic unmyelinated nerve fibres (Fig. 17.). They can be found in large numbers in the layer between the pars tuberalis and the primary plexus. Similar fibres have not been observed around the portal loops. They consist of one or more axons embedded in a Schwann-cell, and are likely to belong to the vasomotor fibres coming from the plexus caroticus.

Discussion

It is widely known that the median eminence is an area of major importance in the hypothalamus-hypophysis interaction. Both physiological and experimental morphological data indicate that the hormone production of the adeno-hypophysis is controlled by the hypothalamus. This control suggests a transmission of informations which, in principle, may take place in two ways: by humoral or by neural mechanism. An increasing number of anatomical, physiological, and biochemical data support the former possibility. Our present observations supply data from the submicroscopic domain, and make more obvious the close humoral relation existing between the nerve terminals of the median eminence and the portal vessels.

(1) The area of the median eminence is the only part of the central nervous system in which the cerebral surface is composed not only of glial and ependymal feet, but also of a compact system of numerous axon endings interposed between them.

(2) These endings contain a large number of vesicles, a part of which is filled with a dense substance. The other part is empty and resembles synaptic vesicles.

(3) The endothelium of the capillaries (that of most capillaries of the Mantelplexus and portal vessel loops) is remarkably thin and fenestrated. Such capillaries are characteristic for those parts of the organism where an intensive liquid transport is taking place: i.e. the kidney, intestinal villi, endocrine glands, salivary glands, choriocapillary layer of the eye, etc. It is even more characteristic that the attenuated, fenestrated endothelium always

faces the nerve terminals. The capillaries in that area of the median eminence where there are no axon terminals with secretion (e.g. subependymal vessels, lateral parts of the median eminence) show a normal structure.

On the other hand, few submicroscopic data support the possibility of a neural transmission. We have failed to find fibres emerging from the median eminence and running towards the adenohypophysis to innervate it. Sections of nervous elements, lying in the connective tissue ("contact") space and showing the same internal structure as the axon terminals in the palisade zone can hardly be regarded as hypothalamic fibres innervating the adenohypophysis. Their faded outlines, internal structure, and vicinity to the surface of the median eminence indicate that they are most likely tangential sections of the palisade zone. Fibres, having the same internal structure and penetrating the pars tuberalis to innervate it have not been observed. Supposing that such innervation does occur [29], its frequency and importance can only be considered comparatively insignificant. Distinction must be made, however, between vasomotor innervation and the problem discussed above. Characteristic unmyelinated nerve fibers showing the well known picture of several axons embedded in the Schwann-cell, can frequently be found in this area, particularly around the arterioles of the primary plexus. They are unmyelinated fibres of peripheral origin, which, in all probability, are vasomotor nerve fibres derived from the carotic plexus [5, 14, 15, 20].

Another interesting problem is, to what neurons do the axon terminals belong in the palisade zone. An increasing number of data support the idea that the nerve endings here do not originate, or originate not exclusively, from the paraventricular or supraoptic nucleus, but they are more or less independent of this system [14, 19, 20, 26, 27]. Experimental morphological data [10] are also in favour of the suggestion that, at least in the sparrow, the two systems: the system of the fibres ending in the median eminence and the fibers terminating in the neurohypophysis can be separated from one another. With the help of Golgi's impregnation technique, SZENTÁGOTHAI [28, 29] recently succeeded in demonstrating that the thin fibres terminating in the palisade zone of the median eminence originate from the arcuate and anterior periventricular nucleus of the hypothalamus. Our electron microscopic results seem to support the view of the latter author. One must consider namely that the irregular sections of the axon terminals well correspond to the grape-like nerve endings obtained by the Golgi method; furthermore, that the thin axons, which, on the basis of the Golgi pictures, belong to the parvicellular system (n. arcuatus, periventricularis anterior), can be found in overwhelming majority just over the palisade zone.

The small empty vesicles observed in the nerve terminals are similar to the synaptic vesicles. Pure electron microscopic observations are unable to decide whether or not the content of the vesicles is chemically identical

with that or of the synaptic vesicles. Provided the small empty vesicles would represent true synaptic vesicles, which are supposed to contain acetylcholine, it can be presumed that, on an adequate nervous stimulus, the acetylcholine set free from the vesicles increases membrane permeability and releases the substance from the dense-core vesicles in direction of the capillaries [3, 13, 25, etc.]. The association of the "synaptic" vesicles with the free cell membrane which has already been observed by OOTA [22] is an extremely interesting phenomenon but its importance remains to be clarified. A further question to be studied is whether the dense-core vesicles correspond to the classical neurosecretory granules or they represent cathecolamine granules? They seem to differ in some respects from the neurosecretory granules as described in the neuro-hypophysis. They are smaller in diameter, their content is finely granulated or filamentous and less dense. A further fact to be considered here is that the palisade zone in the rat can hardly be stained with the Gomori chrome-haematoxylin or the aldehyde-fuchsin staining. On the other hand, an intensive reaction for monoamines (cathecolamines?) could be obtained by the fluorescence method [8].

There is hardly any morphological observation which could clear the problem how and in what form are active substances released from the axon endings. We have failed to find dense granules or a dense substance in the extracellular space between the nerve endings and the capillary. It must be thought therefore that active substances are set free from the axon endings in a rather small molecular, soluble form. The two basement membranes in the route of passage of substances also favour this idea.

The role played by the ependyma in the special function of the median eminence is not quite obvious. Although there are no reliable signs referring to secretion it must be noted that the presence of numerous lipid droplets can be followed from the cell body through the basal process into the ependymal feet. According to the observation of one of the authors [30], lipid droplets are particularly characteristic for the ependyma of the median eminence. It cannot be excluded that in addition to their supporting and trophic function, the ependymal cells of the median eminence play a special secretory role.

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ELEKTRONENMIKROSKOPISCHE UNTERSUCHUNG DER EMINENTIA MEDIALIS DER RATTE

Es wurde die Feinstruktur der Eminentia medialis der Ratte untersucht. In den Zellen der Ependymschicht kann man einen mäßig entwickelten Golgiapparat, Mitochondrien, Ergastoplasma und freie Ribosomen ebenso finden wie eine große Anzahl von Fetttröpfchen. Ähnliche Tröpfchen können auch in den Fortsätzen der Ependymzellen, die sich zur Gehirnoberfläche erstrecken, und in den Ependymfüßchen festgestellt werden. Die Schicht unter dem Ependym ist reich an Gliazellen. In einigen dieser Zellen können dichte Körperchen in der Größe ähnlich den Mitochondrien beobachtet werden, welche den Gomori-positiven Granula ähnlich sind. Letztere kann man mit dem Lichtmikroskop in diesen Zellen finden. Die Faserschicht ist aus dicken (0,5 bis 1 μ) und dünnen (0,08 bis 0,5 μ) Nervenfasern aufgebaut. Die Außenzone (Palisadenzone) besteht aus Nervenendigungen, welche zwei Arten von Vesikeln enthalten. Die kleineren, elektron-translucenten Vesikel, 240 bis 400 Å im Durchmesser, ähneln synaptischen Vesikeln, während die größeren Vesikel (450 bis 1250 Å) einen dichten Inhalt (dense-core vesicles) haben. Die Letzteren sind den neurosekretorischen Elementargranula ähnlich. Die Kapillaren der portalen Gefäße sind fenestriert. Die Poren der Endothelzellen liegen den Nervenendigungen der Palisadenzone gegenüber. Im Kontaktgebiet zwischen der Oberfläche der Eminentia medialis und der Pars tuberalis können marklose Nervenfasern gefunden werden. Es sind vermutlich vasomotorische Fasern aus dem Plexus caroticus. Die Natur der Nervenfasern der Eminentia medialis und der Mechanismus der Abgabe der neurosekretorischen Substanz werden diskutiert.

ЭЛЕКТРОННОМИКРОСКОПИЧЕСКОЕ ИССЛЕДОВАНИЕ СРЕДИННОГО ВОЗВЫШЕНИЯ В ГИПОТАЛАМИЧЕСКОЙ ОБЛАСТИ У КРЫС

Исследовалась ультраструктура срединного возвышения гипоталамической области у крыс. В клетках эпендимального слоя можно выявить среднеразвитый аппарат Гольджи, митохондрии, эргастоплазму и свободные рибосомы, а также большое количество липоидных капелек. Подобные капельки обнаруживаются также в отростках эпендимальных клеток, идущих к поверхности мозга и в эпендимальной ножке. В слое под эпендимой имеется множество глиозных клеток. В некоторых из них наблюдаются плотные тела подобного размера как и митохондрии, напоминающие Гомори-положительные зернышки, обнаруживаемые в этих клетках под микроскопом. Фиброзный слой состоит из толстых (0,5 — 1 μ) и тонких (0,08 — 0,5 μ) нервных волокон. Внешняя зона (палисадный слой) образуется из нервных окончаний, содержащих два типа пузырьков. Меньшие пузырьки диаметром в 240—400 Å, просвечиваемые в электронном микроскопе, подобны синаптическим пузырькам, в то время как более крупные пузырьки (450—1250 Å) имеют плотное содержание (плотно-ядерные пузырьки). Последние напоминают невросекреторные зернышки. Капилляры воротных сосудов окончаты. Поры эндотелиальных клеток обращены к нервным окончаниям палисадного слоя. В смежной области между поверхностью срединного возвышения и pars tuberalis встречаются безмякотные нервные волокна, которые предположительно являются сосудодвигательными волокнами, идущими от сонного сплетения. В статье обсуждаются природа нервных волокон в срединном возвышении гипоталамической области и освобождающий механизм невросекреторного вещества.

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Acta Biol. Hung., **15**(4), 459—464 (1965)

LAMELLAR BODIES IN PURKINJE CELLS OF THE GUINEA PIG

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(Received November 6, 1964)

Synopsis

Purkinje cells in the cerebellar cortex of the guinea pig contain electron microscopically demonstrable lamellar bodies, similar to those already described by other authors in the Purkinje cells of the rat. It is assumed that lamellar bodies reflect some functional state of the neuron.

Introduction

In 1957, FERNÁNDEZ-MORÁN described [1] lamellar structures in the Purkinje cells of the rat, that were further studied using the perfusion technique of PALAY and designated as "lamellar bodies" by HERNDON [5].

In our sections obtained from samples fixed by the simple immersion method, we repeatedly observed these structures in Purkinje cells of guinea pigs. It appears, therefore, that lamellar bodies are common constituents of Purkinje cells.

Material and method

The vermis of the guinea pig has been used for these studies. Samples were obtained by neurosurgical methods under Evipan anaesthesia. Fixation has been performed according to the technique described by MILLONIG, dissecting the sample in the osmic acid solution to obtain blocks measuring about 1 cubic millimeter. After fixation (one hour) and a successive dehydration in an alcohol series, the samples were embedded in Araldite. Silver sections obtained on an LKB Ultrotome, using glass knives, were placed on Formvar membranes and stained by means of the REYNOLDS [7] lead citrate solution. The sections were examined and photographed in the JEM 6C electron microscope at the Department of Morphology, Institute of Experimental Medicine, Hungarian Academy of Sciences (Budapest).

Results

In the cytoplasm of the Purkinje cells a well defined system of the endoplasmic reticulum can be observed (Fig. 1). At most places, the membranes of the reticulum are covered with RNP granules, 150 Å in diameter. These particles can also be observed scattered throughout the cytoplasm. Other components of the hyaloplasm described earlier by a number of investigators are also present; vesicles, multivesicular bodies, granules and mitochondria

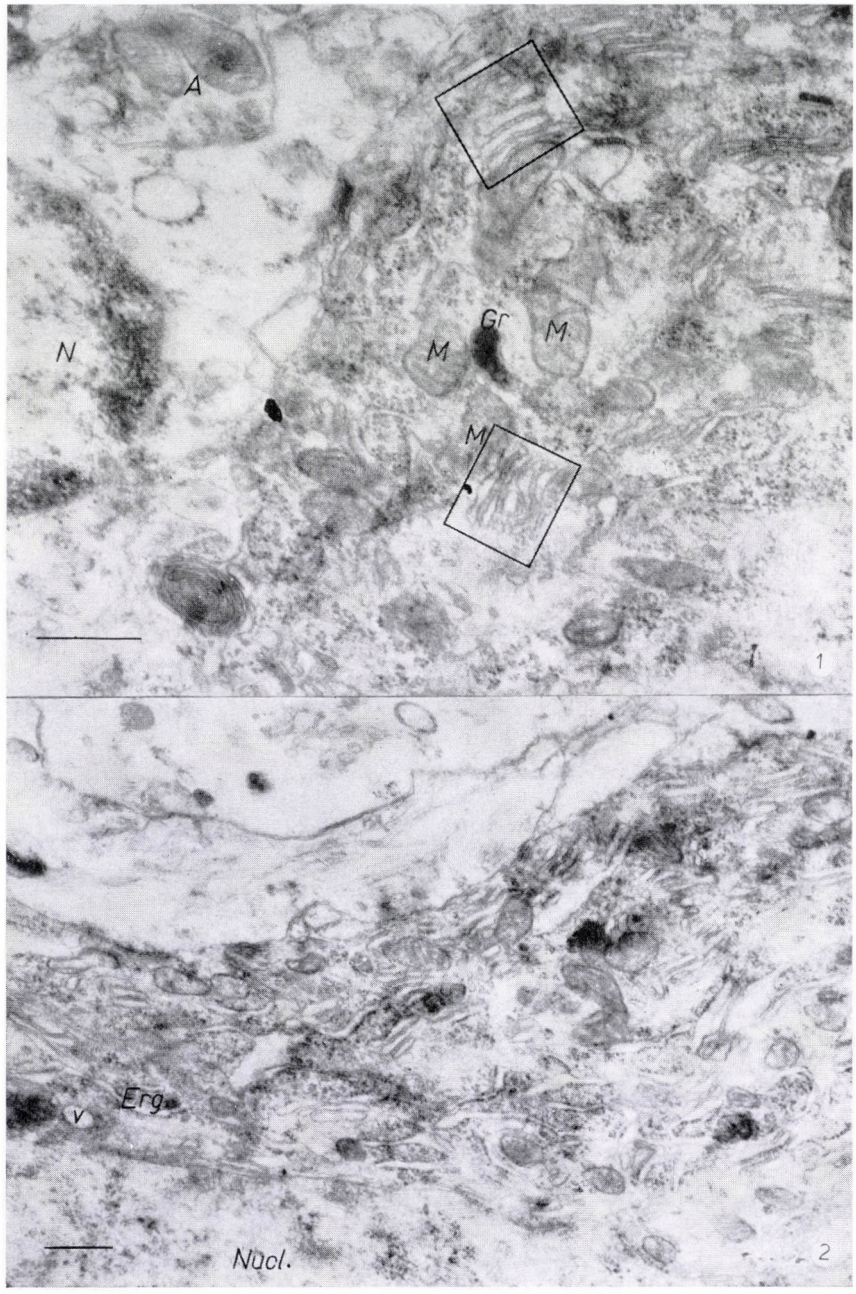

Fig. 1. Purkinje cell of the guinea pig. Lamellar bodies are outlined. Gr = granule, M = mitochondrium, N = glial nucleus, A = axon. × 14,000

Fig. 2. Purkinje cell of the guinea pig. Lamellar bodies in the rectangles. Erg. = ergastoplasm (granulated reticulum), V = vesicles, Nucl = nucleus of the Purkinje cell. × 9,000

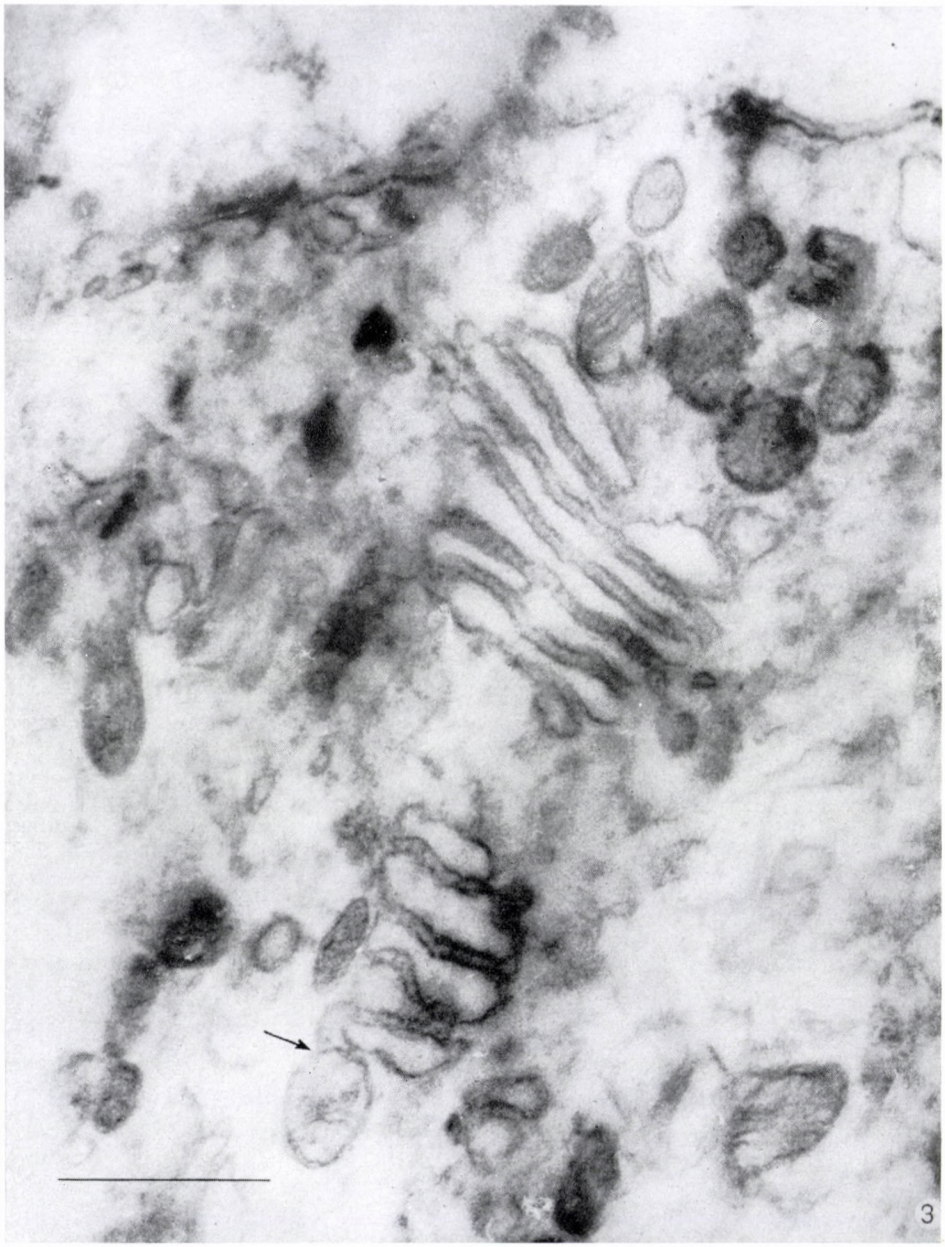

Fig. 3. Lamellar bodies in the cytoplasm of a Purkinje cell. Arrow points to a connection (?) between a lamellar body and a mitochondrium. × 28,000

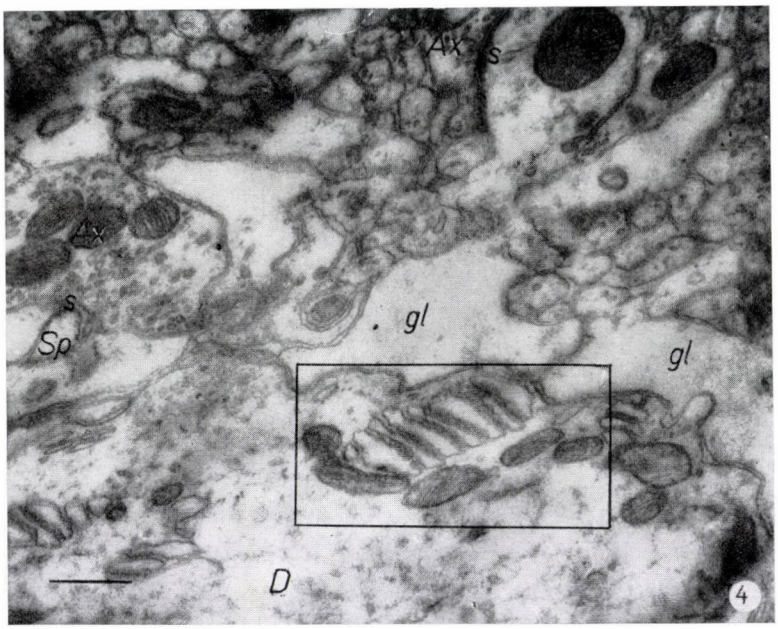

Fig. 4. Primary dendrite of a Purkinje cell (guinea pig). Cross-section of the dendrite. A lamellar body, in the vicinity of mitochondria, appears in the rectangle. D = dendrite, S = synapse. Ax = axon, sp. = dendritic spine, gl = glial cell. × 9,000

(Fig. 2). On the other hand, even under low power, structures exhibiting a lamellar structure can also be seen. These lamellar bodies consist of sheet-like units oriented more or less parallelly; on the surfaces of these units a system of thin cross-striation can be observed interconnecting neighbouring lamellae, that may be a modifed form of RNP granules but may represent also some more specialized structure. The lamellae deliminate clefts or holes measuring 400—600 Å; these "holes" appear to contain no electrondense deposits. Lamellar bodies can be found both under the surface membrane of the cytoplasm and in the vicinity of the nucleus. Close to the lamellar bodies, mitochondria are aggregated (Figs 3 and 4). Lamellar bodies are found also in the primary dendrites of Purkinje cells. Here these structures are located under the surface of the dendritic branches. Mitochondria surround lamellar bodies in the dendrites, suggesting some special correlation.

Discussion

Our observations prove that lamellar bodies occur not only in the rat as described already, but also in the Purkinje cells of the guinea pig. It is surprising that, in spite of the number of electron microscopic investigations

carried out on cerebella of various species [2, 3, 4, 5, 7], only a few authors observed lamellar bodies. It may be assumed that lamellar bodies appear only in some functional state of the Purkinje cell. According to HERNDON, lamellar bodies are derived from the endoplasmic reticulum, due to transient anoxia, to the pressure of the knife (when collecting the sample) or to the low temperature of the fixing solution. In our opinion the role of anoxia is problematical since, in our studies, the interval between sampling and completing the fixation did not exceed 35—40 seconds. It is difficult to decide whether the low temperature of the osmic acid solution plays any major role since some of the authors who did not observe lamellar bodies [2, 6] also employed cold osmic acid solutions. According to HERNDON [5] in sections obtained from animals artificially respirated till the onset of perfusion with warmed (37°C) osmium tetroxide solution, no lamellar bodies could be found in Purkinje cells. It is worth mentioning, however, that though most of the Purkinje cells in our sections (obtained from samples fixed by the immersion technique) contained lamellar bodies, there were consistently found Purkinje neurons without these structures. It appears therefore, that some functional factor plays an important role in the genesis of the lamellar bodies. Neither can the possibility be excluded that there exist actual structural differences between various Purkinje cells of the same animal.

Acknowledgement

The authors are indebted to Dr. B. CSILLIK for his advices and criticism throughout this work. Our thanks are due to Dr. P. RÖHLICH and Mrs. L. ALPÁRY for their help in the technical work.

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LAMELLENKÖRPERCHEN IN DEN PURKINJEZELLEN DES MEERSCHWEINCHENS

An Hand von elektronenmikroskopischen Untersuchungen beschreiben Verfasser die Anwesenheit von den in anderen Tierarten schon beobachteten sog. Lamellenkörperchen im Zytoplasma der Purkinjezellen des Meerschweinchens. Es wird angenommen, daß diese Körper, die wahrscheinlich von dem endoplasmatischen Retikulum abstammen, einen speziellen funktionellen Zustand der Purkinjezellen andeuten.

ПЛАСТИНЧАТЫЕ ТЕЛА В КЛЕТКАХ ПУРКИНЬЕ МОРСКОЙ СВИНКИ

Клетки Пуркинье в коре можечка морской свинки содержат пластинчатые тела, доказуемые электронномикроскопным методом подобно тому, как клетки Пуркинье крыс. Авторы предполагают, что пластинчатые тела отражают какое-нибудь функциональное состояние неврона.

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Acta Biol. Hung., **15**(4), 465—479 (1965)

THE PURKINJE CELL BASKETS: ULTRASTRUCTURE OF AN INHIBITORY SYNAPSE

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(Received January 30, 1965)

Synopsis

An electron microscope study of the cat cerebellar Purkinje neuron basket. Most part of the Purkinje cell body surface is blocked from immediate contact with other nerve elements by glial plasma. The descending branches of basket neuron axons, unusually rich in neurofilaments, establish synaptic contact with the lower part of the Purkinje cell body and also with what is generally thought to be the initial segment of the axon. Postsynaptic structures in the form of flattened sacs, lying parallel to and immediately beneath the postsynaptic membrane, occur frequently opposite to accumulations of synaptic vesicles. — The basket axon branches — after having established synaptic contacts with the lower part of the Purkinje cell body and its basal process (pre-axon) — are braking up in outward direction in brush-like manner into thin finger-shaped processes, containing few synaptic vesicles. These thin terminal processes establish a loose neuropil surrounding the Purkinje cell basket from the outside. It consists of intricately interwoven thin axonal branches and few thin glial processes, easily recognizable from their dense glycogen bodies. The outer basket neuropil has a very considerable amount of extracellular space, containing a slightly granular matrix. — The possible functional significance of these findings is discussed in the light of recent information on the inhibitory role of the Purkinje basket synapse.

Introduction

With respect to the numerous and characteristically different types of synapses by which the several kinds of its interneuronal connexions are established, the cerebellar cortex offers an unique material for electron microscopic studies of different kinds of synaptic contacts that are established with the same kind of neuron. In addition to this, recent advances in the microphysiology of cerebellar cortical neurons [6, 1] have furnished some direct information concerning their functional properties and some of their synapses. Speculations based on the geometry of neuronal linkage and on some recent quantitative data [3]; and own observations [12] on cerebellar neuron connexions convincingly lead up to the conclusion that the basket synapse must be inhibitory in nature [11]. Direct evidence of the inhibitory character of this synapse has been presented by ANDERSEN, ECCLES and VOORHOEVE [1]. — Thus a detailed investigation into the ultrastructure, not only of the basket synaptic contacts established with the Purkinje cell surface — which in themselves do not seem to be of very particular nature — but of the whole synaptic apparatus, is of considerable interest.

Material and method

The cerebellar cortex of adult cats, exposed under ether anaesthesia, was fixed in situ by local application either of cold buffered osmic acid or isotonic glutaraldehyde solution and small pieces excised by an instrument consisting of four parallel razor blades, arranged at distances of $^1/_2$ mm, were continuously after-fixed in the same solutions. In the case of glutaraldehyde prefixation (one hour) the material was transferred into buffered osmic acid solution for after-fixation. The material was embedded in Araldite, cut on the LKB ultratome and the sections were mounted on grids without films and stained with lead-citrate. — The specimens were examined with the "Tesla" (Brno) table electron microscope.

Results

The results of these investigations are shown semidiagrammatically in Fig. 1. The whole upper part of the cell body as well as primary dendrites are completely enveloped by glial tissue — probably mostly of the Bergmann glia — so that no nervous elements can get into immediate contact with these parts of the Purkinje neuron surface. Only very occasionally club-shaped axon terminals containing synaptic vesicles are admitted to the surface of the main dendrites, where they establish synaptic contacts of Gray's type 2. Their number increases with the distance from the cell body and they are quite abundant on the secondary branches of the main dendrites. It is known from light microscopy that it is mainly the terminals of recurrent Purkinje axon collaterals, which establish synaptic contact with the main dendrites, therefore most of these synapses apparently belong to these collaterals. This fits also very well into the observation that numerous myelinated fibers are visible between the Purkinje cell bodies and immediately above — i.e. towards the surface — which correspond to the so called supraganglional plexus of myelinated fibers, belonging mainly to the same recurrent collaterals. There is, however, another type of axons in close relation to the main dendrites of Purkinje neurons: the climbing fibres, which lose their myelin sheaths at the level of the Purkinje cell bodies. Our attempts — successful to some extent — to distinguish climbing fibers from recurrent Purkinje axon collaterals electron microscopically would lead us, however, far astray, so that these findings will be reported elsewhere.

The descending preterminal basket axon branches are easily recognizable on the basis of their large diameter, their unusually numerous neurofilaments and their position immediately outside the glial envelope of the Purkinje cell bodies. The climbing fibres being, unfortunately, of equally neurofilamentous character, allowance must be made for one or the other of the numerous neurofilamentous fibers which are in reality climbing fibers. The nature of the fibers becomes immediately clear beneath the equator of the cell body where the basket axon branches approach the lower part of the Purkinje cell body and establish synaptic contacts with the lower third and especially the region

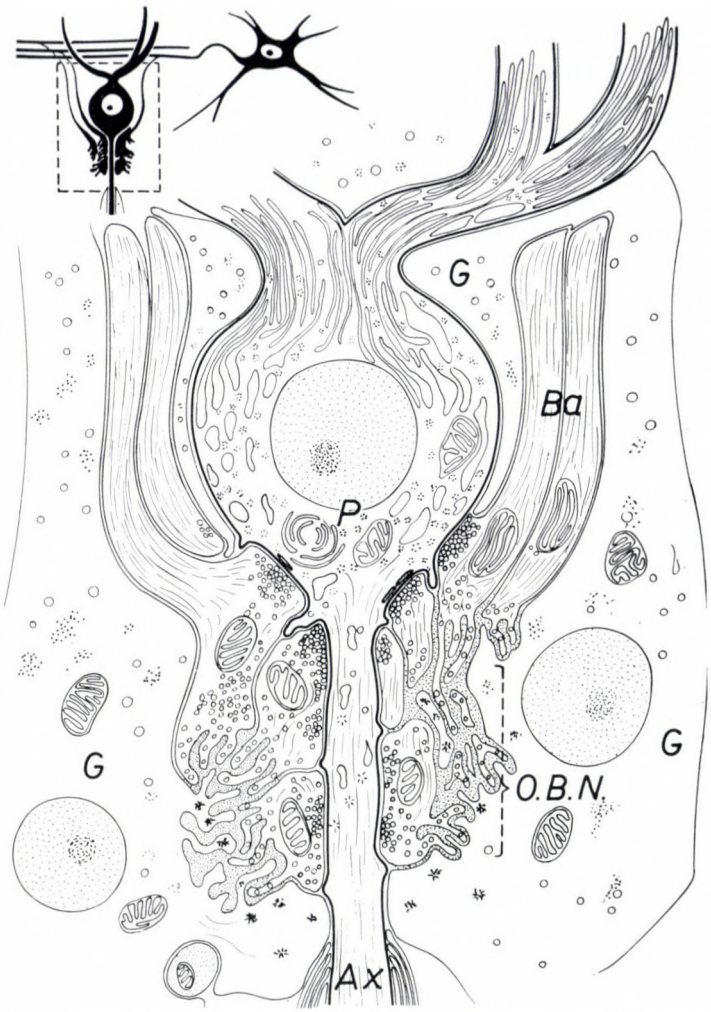
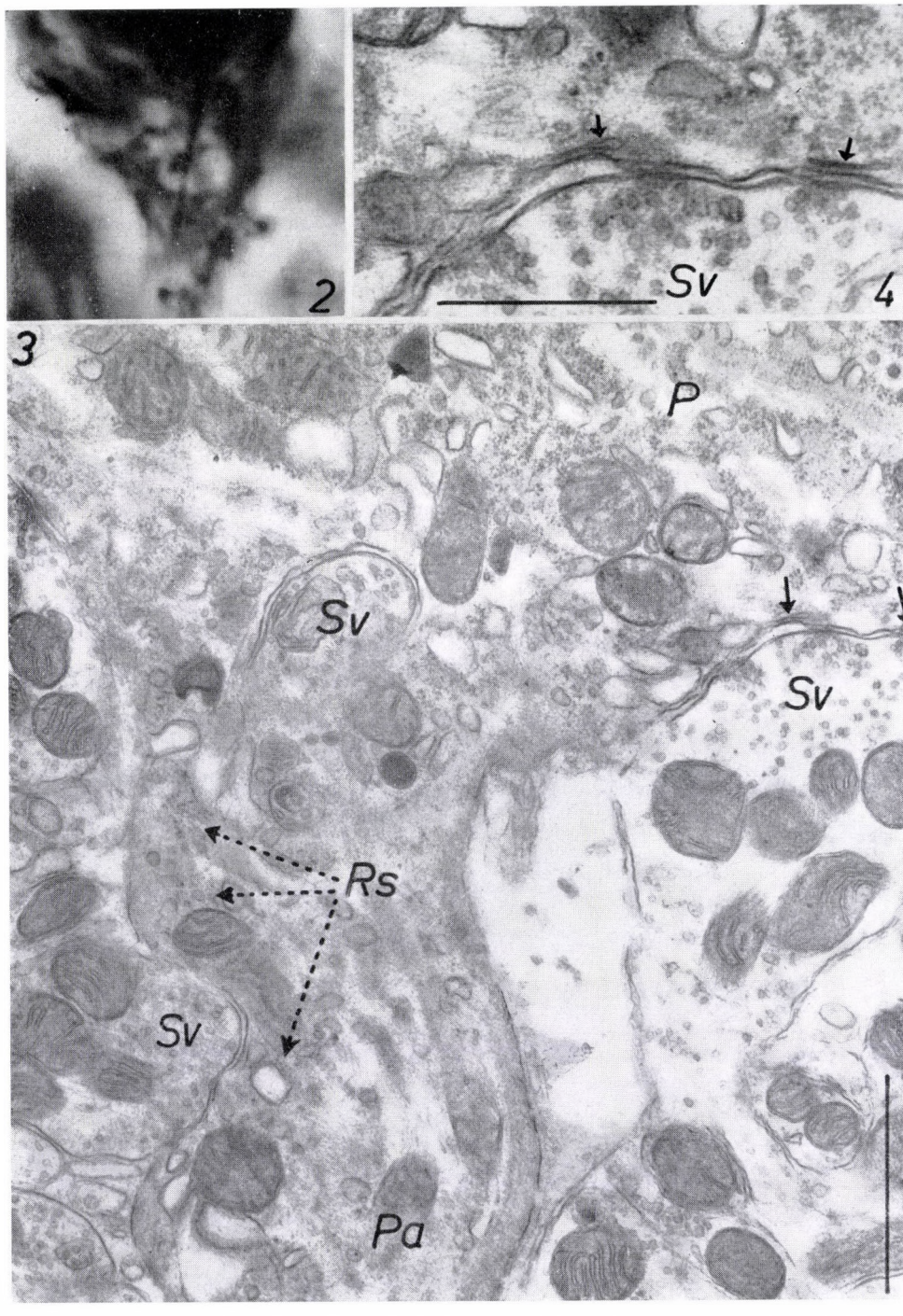


Fig. 1. Diagram illustrating the fine structure of the Purkinje cell baskets in the cerebellar cortex. Small diagram left above with interneuronal relations, as known from light microscopy, shows (dashed rectangle) the region represented in the main diagram. Upper part of Purkinje cell body (P) and main dendrites are almost completely enveloped by glial cytoplasm (G, —mainly of the Bergmann glia). Descending preterminal branches of basket axons (Ba) unusually rich in neurofilaments have synaptic contacts only with the bottom of the cell body, and the basal process of the cell, which according to its structure is not really axonal but could better be labelled as "preaxon". The true axon (Ax) begins deeply downwards in the granular layer. Subsynaptic differentiations in the form of flat sacs opposite to accumulations of synaptic vesicles are frequent in the cell body, however, are lacking in the preaxon. The terminal branches of the basket axons break up in outward direction into finger-like processes, forming an intricate outer neuropil of the baskets (OBN) with similar processes of the glial cells. Wider intercellular spaces of this region are indicated by dotting. — The orientation of all following electron micrographs corresponds to this diagram


2
Sv
4
3
P
Sv
Sv
Rs
Sv
Pa

of the "axon hillock" (Figs 3, 6, 7). The cell surface in this region is very rugged, having deep impressions into which some of the presynaptic regions of the axons are embedded. Between the impressions the cytoplasm of the Purkinje cell appears to protrude far outwards (Fig. 6). In tangentially cut parts of the cell body one often sees axonal profiles surrounded on all sides by the cytoplasm (Fig. 3). Most part of the axonal profiles is filled with neurofilaments and generally only the regions of immediate contact contain rather moderate amounts, or sometimes even few of the usual synaptic vesicles (Figs 6, 7, 8). Dense bars can be seen frequently immediately beneath the postsynaptic membrane (Fig. 6). At some places (Fig. 4) it becomes clear that these bars are in fact flattened sacs, known already and considered as specific postsynaptic structures in several other interneuronal synapses.

The basket axon branch does not, in most cases, terminate after having established one or two synaptic contacts with the lowest part of the Purkinje cell. Most of the synapses with the cell body are only contacts "de passage" and the preterminal axon continues its descending course towards the granular layer (Figs 7, 8). This is clearly recognizable also under the light microscope in silver stained preparations (Fig. 2), an arrangement often described and illustrated in the classical descriptions of the Purkinje cell baskets. As seen from Fig. 2 the "initial segment" of the Purkinje "axon" is surrounded for a considerable distance — generally of about 10—30 microns and more — by a dense envelope of basket axon branches. In accordance with this in the electron microscope the initial part of the Purkinje "axon" is always surrounded by some large profiles of terminal axon branches (Figs 3, 5). Whereas the number of synaptic vesicles is generally small in the regions of contact with the bottom of the cell body, the lower ends of the same basket axons are rather filled with them. The axonal endings are often fairly deeply impressed into the surface of the single cell process that originates from the bottom of the cell, which could be identified easily with the axon. Synaptic contacts occur between the lower ends of the basket terminals and these processes (Figs 3, 5). One might therefore consider these contacts as axo-axonic if it were not for the strange non-axonal structure of the basal process of the Purkinje cell. Although this process contains numerous neurofilaments, the presence of ribosomes and

Fig. 2. Light microscopic picture of the Purkinje cell basket. The basal cell process or pre-axon can be seen in the center of the descending part of the basket. Cat, Dejnek method, magnification ×2,000

Fig. 3. Basal part of Purkinje neuron (P) with several basket axon endings, containing synaptic vesicles (Sv) attached to bottom of cell body and to basal process or pre-axon (Pa), in which neurofilaments, endoplasmic sacs and ribosomes (Rs) are visible. In upper center a tangentially cut basket axon ending can be seen embedded into bottom part of the cell; subsynaptic differentiations shown enlarged in Fig. 4 are indicated by arrows. Scale 1 micron

Fig. 4. Enlarged part of Fig. 3. Arrows indicate flat synaptic sacs situated opposite accumulations of synaptic vesicles. Scale 0.5 micron

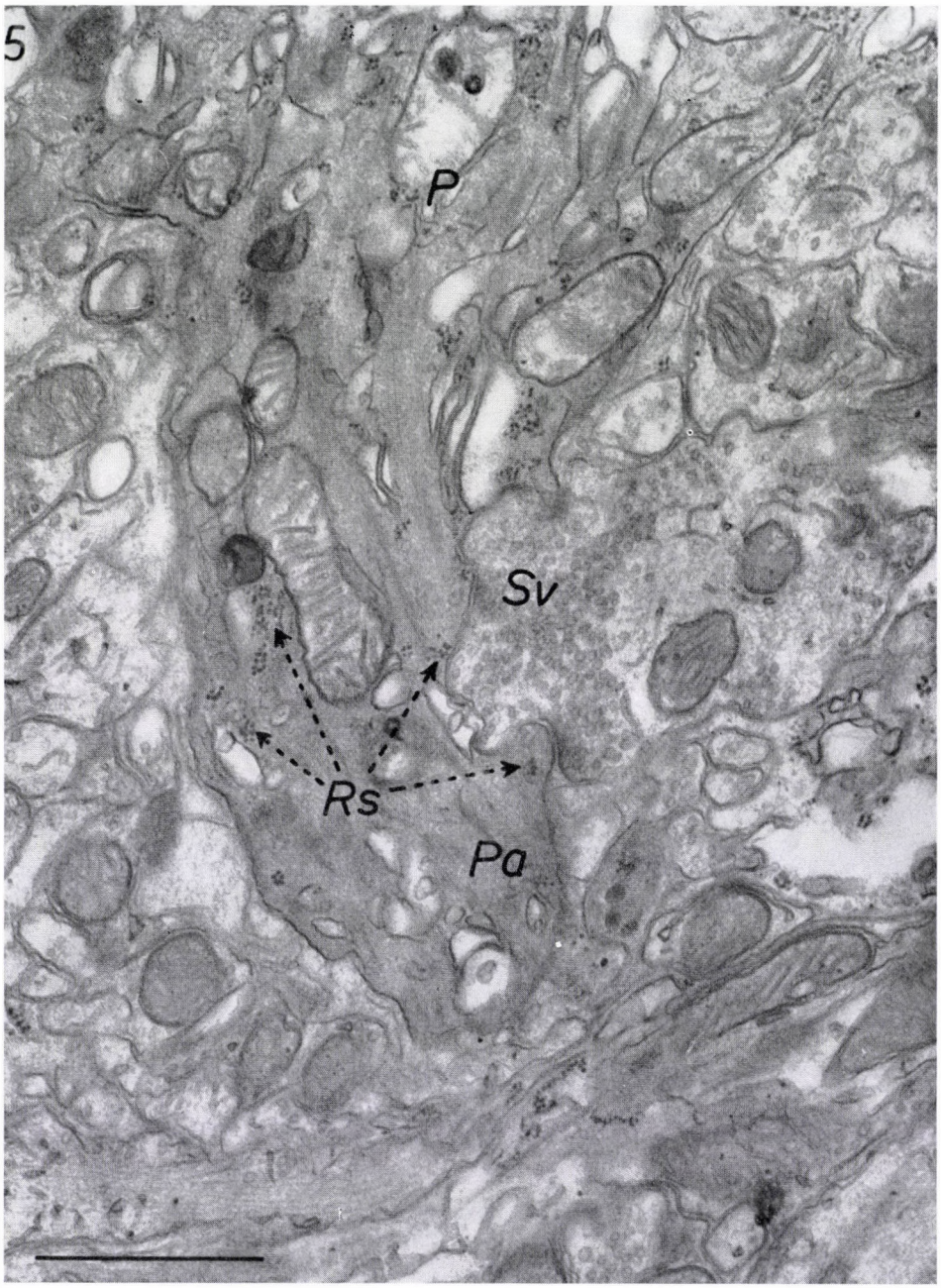


Fig. 5. General view of the origin of basal process or pre-axon with part of the Purkinje cell body (P) and large profile of basket axon ending closely attached to pre-axon (Pa). The basket ending contains transversally cut neurofilaments at the far side and synaptic vesicles (Sv) near the region of attachment. Specific accumulations of vesicles are seen at two places of the contact. Neurofilaments, endoplasmic sacs and ribosomes (Rs) are seen everywhere in the pre-axon (Pa). Scale 1 micron

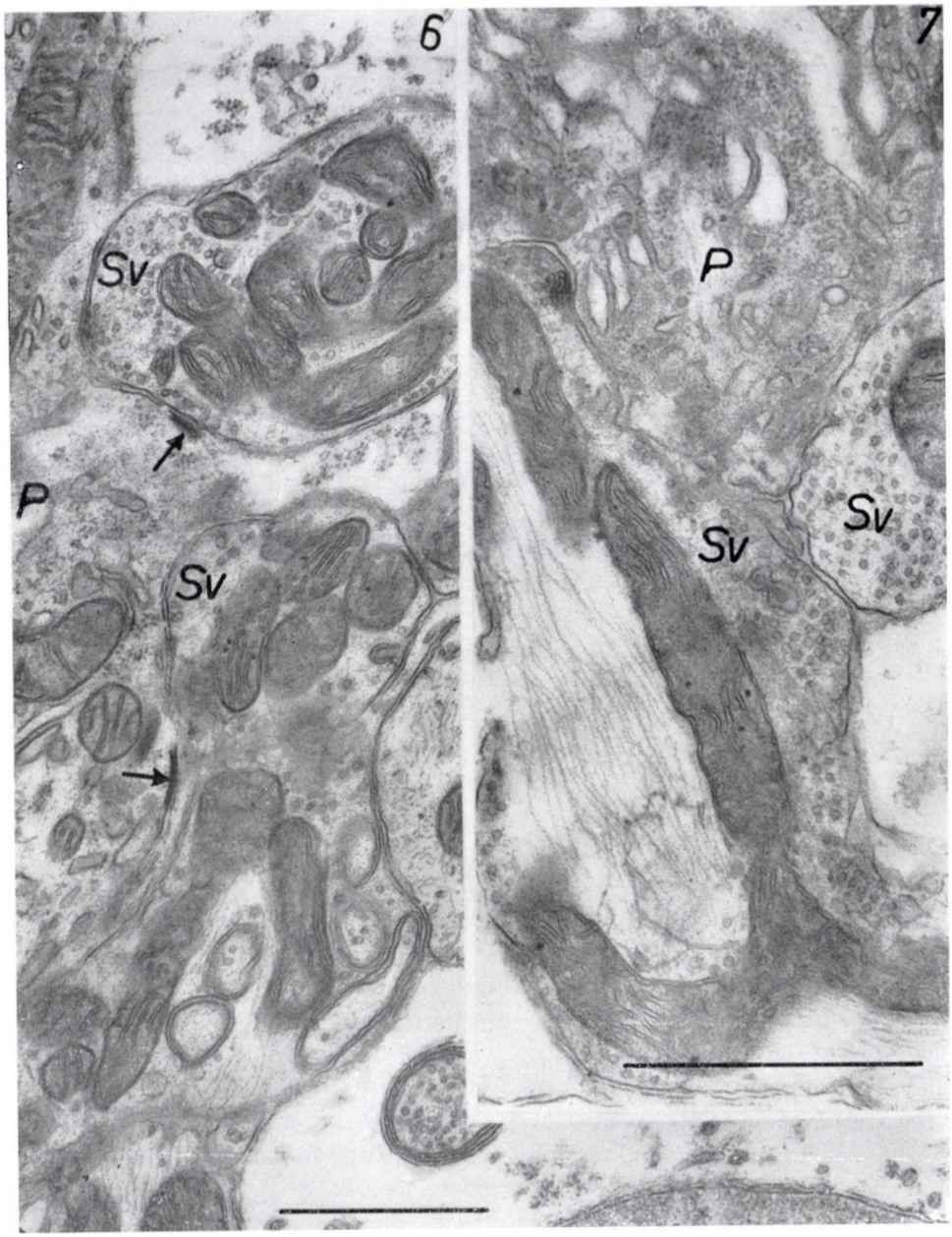


Fig. 6. Two basket endings containing but few synaptic vesicles (Sv) attached to Purkinje neuron surface (P) with subsynaptic flattened sacs (arrows); a pseudodendritic protrusion of Purkinje cell plasma separates the presynaptic endings. Neurofilamentous invaginations in lower presynaptic profile are indicating probably the breaking up of the large ending into the finger-like processes of the outer basket neuropil. A bottom right nucleus of Bergmann glia and embedded preterminal axon. Scale 1 micron

endoplasmic reticulum (Fig. 5) would favour a somewhat different interpretation.

The bulbous enlargements of basket axon branches are finally breaking up in outward direction into numerous small (0.1—0.2 micron diameter) finger-like processes, containing few synaptic vesicles, which contribute to a strange loose neuropil that peripherically surrounds the Purkinje baskets (Figs 9, 10). This peripheral loose neuropil of the Purkinje cell baskets is built up of these finger-shaped processes of the basket axon terminals and glial processes of equal size — of the Bergmann glia cells — containing characteristically dense glycogen bodies (Figs 9, 10). Strangely, this neuropil contains no clear synaptic contacts at all. Whereas the larger profiles of the basket axon terminals are fitted together rather tightly, or their interstitial spaces are filled up by glial profiles, their small processes in the outer neuropil are separated from each other by wide intercellular spaces. As seen in Figs 9 and 10 this intercellular space — quite unusual otherwise in the CNS — is filled up by a slightly granular matrix. At any rate the glial profiles look more empty, in general, than the intercellular space.

Discussion

This synaptic arrangement is of considerable interest in more than one respect. First of all it is the high complexity of the apparatus that is most remarkable. Although light microscopy has already clearly shown that it is by no means simply an axosomatic synapse, in the form of a dense pericellular basket, but an arrangement that involves additionally also what was thought to be the initial segment of the Purkinje axon (Fig. 2), the situation is still more complex in reality. The basket axon terminals have undoubtedly synaptic terminals with both the bottom of the cell body — what in the case of other neurons would be the axon hillock — and also with the single process arising from the lower pole of the Purkinje cell. The finer structure of this process however, differs characteristically from true axons which do not in general contain ribosomes and endoplasmic reticulum. It might therefore be more appropriate to consider the initial part of this basal process as a thin elongation of the cell body (pre-axon), which is "drawn out" for about 10—20 microns, whereas the true axon would begin only deeper in the granular layer. — It has been mentioned by several authors — and under the light microscope it really appears so — as if some of the mossy fibers or their collaterals might

Fig. 7. Synaptic basket axon terminals attached to bottom (probably site of origin of the pre-axon) of Purkinje cell body (P), (note neurofilaments). Left terminal, very rich in neurofilaments and rather poor in vesicles, is seen to descend further towards lower part of basket. Scale 1 micron

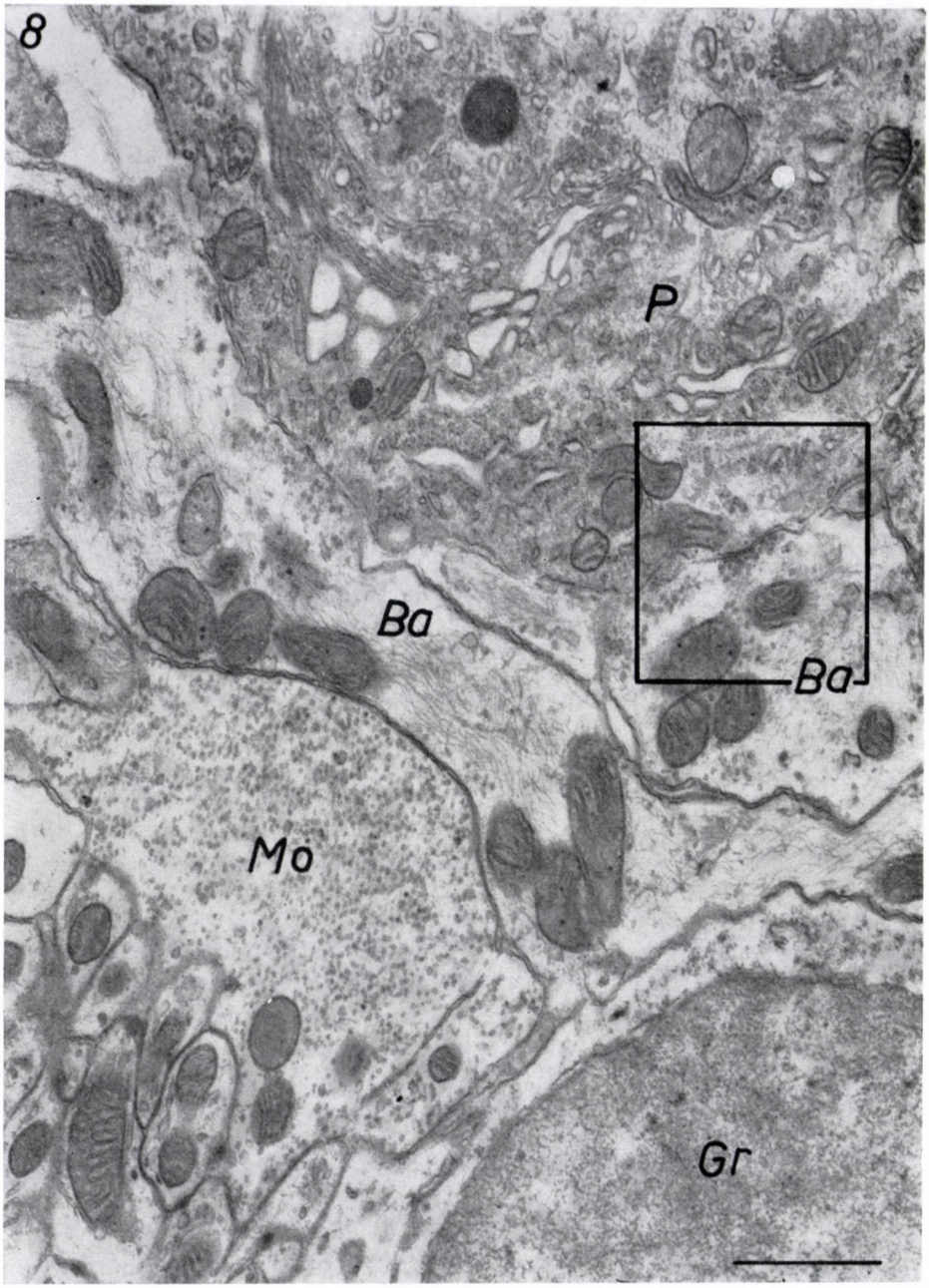


Fig. 8. Bottom of Purkinje cell body (P) with two descending basket axons (Ba) attached. Although mossy terminal (Mo) and granule cell (Gr) are closely attached for long distance to outer surface of basket axon, there is no indication whatever of specific synaptic differentiation in either contact. Nor has the mossy fiber any differentiations in its accidental contact with granule cell. The mossy ending has specific differentiations only with ends of granule dendrites, lower left, not clearly visible here because of tangential direction of the plane of section. Scale 1 micron. Framed part corresponds to Fig. 11

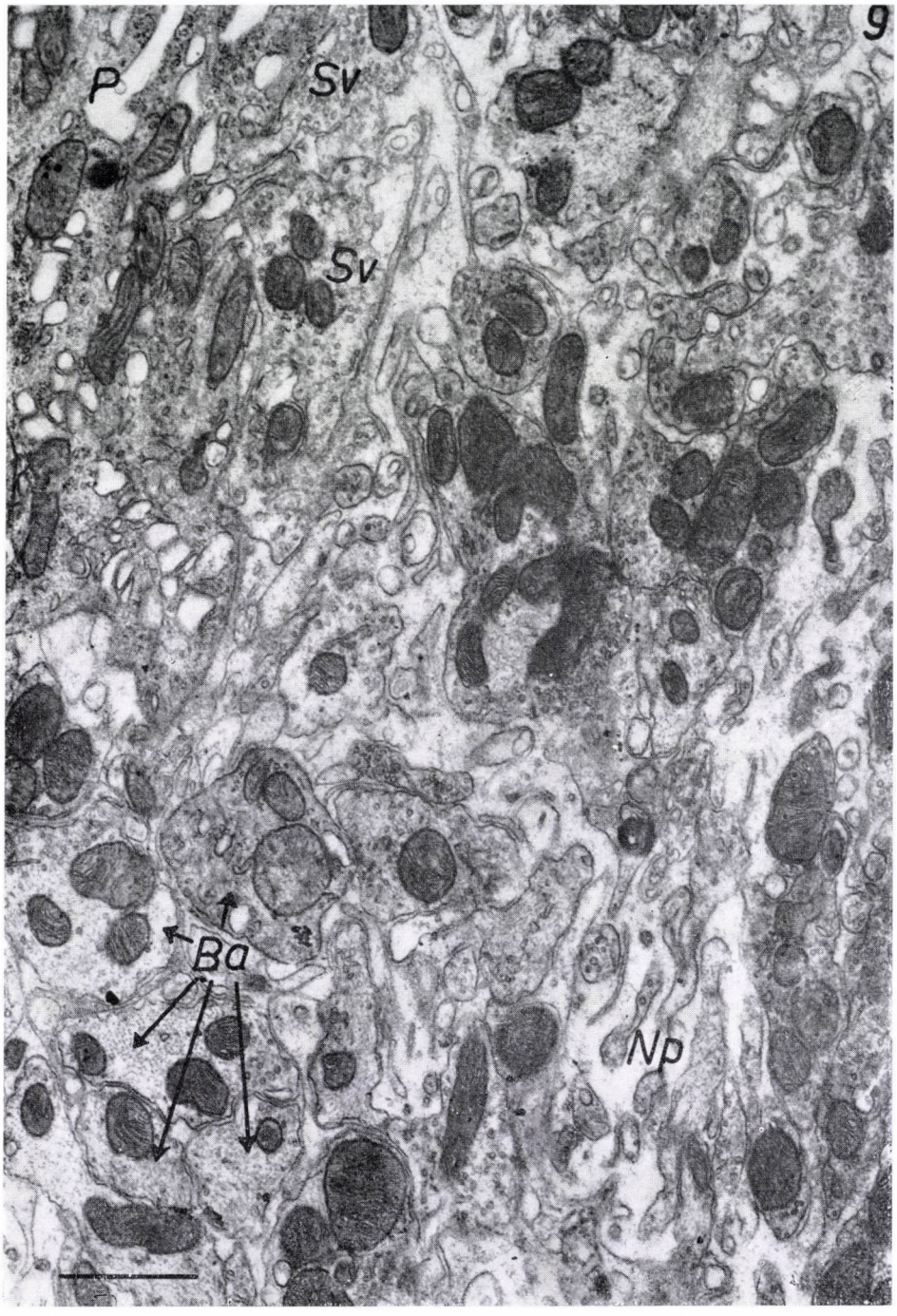
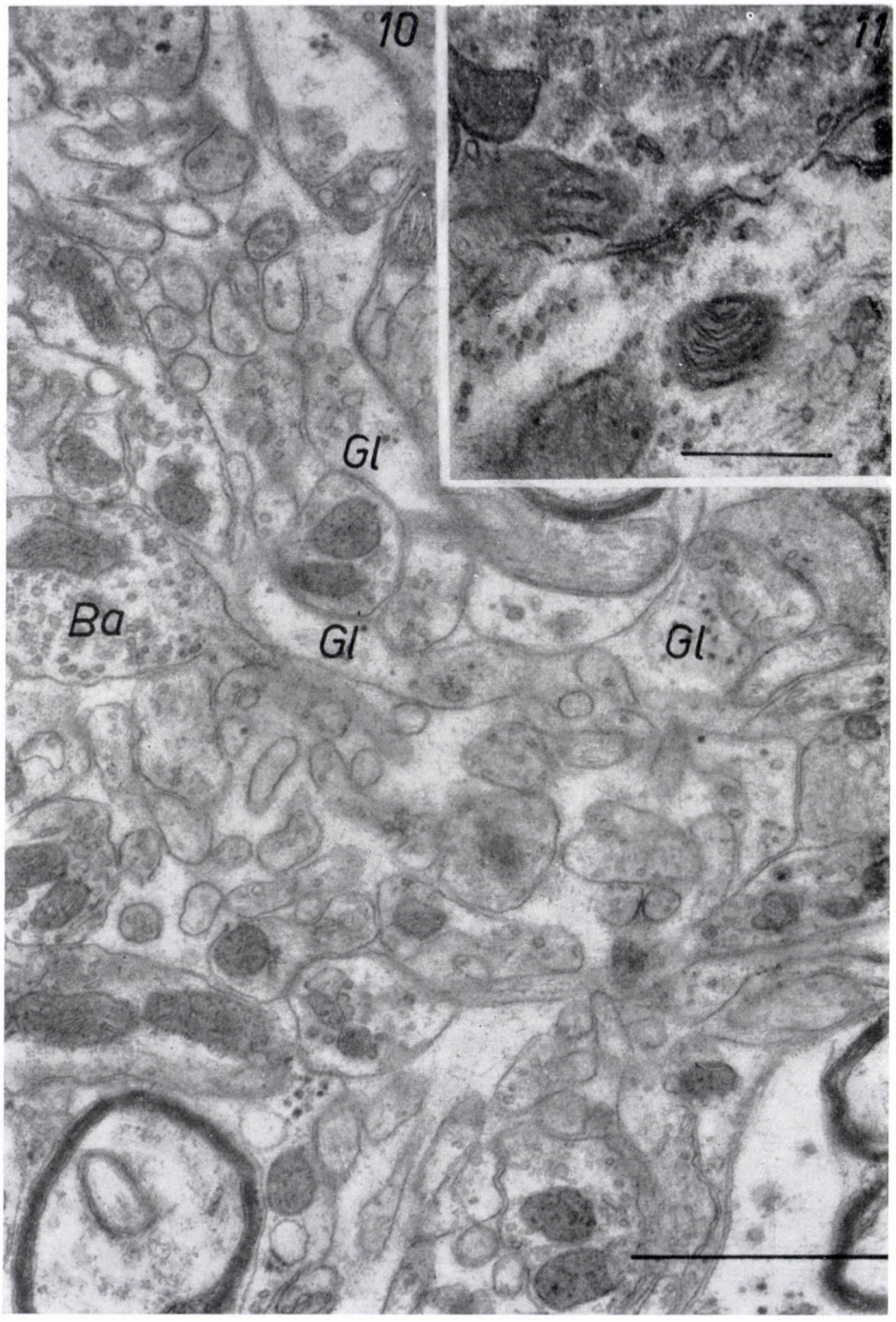


Fig. 9. General view of Purkinje cell basket with small magnification. Bottom part of Purkinje cell body (P) is seen at upper left with basket terminals attached, synaptic vesicles (Sv) containing. Below numerous neurofilamentous basket axons (Ba) from the lower beard-like (see in the light microscopic picture, Fig. 2) part of the basket. The outer part of the basket is a looser neuropil (Np) built up of very thin processes, both glial and axonal, with unusually large amount of intercellular space. Scale 1 micron


10
11
Gl
Ba
Gl
Gl

get into contact with the baskets [7]. As seen in many of our own preparations (Fig. 8), mossy terminals may be closely attached to the outside of the baskets, without showing, however, the slightest indication of specific synaptic contact differentiations. The same mossy fiber has numerous specific contacts with granule cell dendrites on its opposite side. The few dendritic profiles encountered in the outer part of the outer basket neuropil having no synaptic contacts with its elements either, must be interpreted as accidentally involved in the outer region of the basket.

Another remarkable feature of the basket synapse is the strange loose neuropil formation surrounding the inner baskets. One may argue that the origin of the fine axonal elements from the lower ends of the basket axon terminals is not clearly established. A comparison with silver stained preparations under the light microscope and especially also with pictures showing secondary degeneration of the baskets [12], suggest that the whole basket is built up exclusively of basket axon terminals. Whereas the larger axon terminals immediately surrounding the bottom of the cell body and the initial segment of the basal process (pre-axon) are fairly tightly fitted, the finger-shaped outer branches of the axons, interdigitating with glial processes of similar size, are unusually loosely packed with considerable intercellular spaces. One might again object that this could be an artifact. We do not want to enter into the discussion, whether or not intercellular spaces in central nervous tissue are artifacts. Intercellular spaces of this kind did not occur in our preparations of the cerebellar cortex, with the exception of the outer part of the baskets and around certain dendrites of Golgi cells. The presence of glycogen bodies in the glial processes shows that there was no major tissue anoxia, often mentioned as being the cause of larger intercellular spaces. The interstitial space contains a granulated material of somewhat larger density than the glial profiles.

The unequal distribution of synaptic vesicles is interesting too. The axon branches neighbouring the bottom of the cell body are extremely rich in neurofilaments and poor in synaptic vesicles. Only small groups of vesicles are encountered at the sites of true synaptic attachments opposite to regions equipped with the flattened postsynaptic sacs (Figs 3, 4, 6). The filaments become more sparse and the vesicles more abundant in the lower synaptic terminals attached to the pre-axonal Purkinje cell process. There are still

Fig. 10. Tangential section through outer basket neuropil, with somewhat larger glial (Gl) and smaller axonal profiles. A small finger-shaped axonal process is seen to originate at the right side of large basket axon (Ba) terminal. No synaptic differentiations occur in this neuropil. The few dendritic profiles (upper right and lower left) are parts of the characteristically curved granule neuron dendrites, which do not participate in the baskets and turn back to surrounding glomeruli. Scale 1 micron

Fig. 11. Enlarged part of Fig. 8 with specific synaptic attachment — of Gray type 2 — between Purkinje cell body and basket axon. Scale 0.5 micron

a few vesicles but no filaments in the finger-shaped outer processes of the axon-endings. If it were true that the synaptic vesicles are the structural substrates carrying the synaptic mediators, this distribution might indicate that the lower axon endings attached to the pre-axonal process are the more effective ones for transmission. However, the upper endings attached to the bottom of the Purkinje cell body have much clearer postsynaptic differentiations, so that these attachments can by no means be interpreted as merely accidental. — No explanation at all can be given for the outer loose neuropil of the baskets, lacking completely any specific regions of synaptic contact.

The Purkinje cell baskets being one of the few inhibitory synapses that can clearly be identified by physiological means [1] it is worth while to attempt to correlate some of the structural features to its function. There is first the localization of the synapse on the bottom region of the cell body and even on the basal cell process or "pre-axon", which is most easily reconciled with its inhibitory function. GESELL [5] was the first to put forward the idea, that the best strategic localization of an inhibitory synapse would be in the region of the axon hillock or the initial segment of the axon. That this cannot be generalized, follows clearly from the occurrence of excitatory synapses on the axon hillock: as e.g. LENHOSSÉK's [8] calyciform synapse surrounding the axon hillock of ciliary ganglion cells of birds and reptiles, which being the only synapse on this neuron, is undoubtedly of excitatory function. On the other hand there are inhibitory synapses on numerous neurons having no peculiar synaptic arrangement in the axon hillock region or around the initial segment of the axon, as e.g. motoneurons. The only synapses known hitherto that meet the requirements of GESELL's hypothesis are the Purkinje basket synapse and the axon cap synapse of the Mauthner neuron. The inhibitory nature of the latter synapse has been postulated by RETZLAFF [9] and the recent analysis by FURUKAWA and FURSHPAN [4] has shown that the synapse exercises inhibition by electrical transmission. Especially from the viewpoint of electron microscopy there is some superficial resemblance between the Mauthner neuron axon cap [10] and the basket synapse but the neuropil of the Mauthner cell axon-cap is extremely dense, whereas that in the outer part of the Purkinje cell baskets, on the contrary, is loose. — The consequences of the inhibitory nature of the Purkinje basket synapse for our understanding of the functions (on larger scale) of the neuronal network in the cerebellar cortex and deductions leading up to a new concept of the higher order integrative units of the cerebellum have been treated *in extenso* already in earlier papers [11, 12].

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DIE PURKINJE-ZELLKÖRBE: ULTRASTRUKTUR EINER HEMMUNGSSYNAPSE

Elektronenmikroskopische Studie der Purkinje-Zellkörbe des Kleinhirns der Katze. Der größte Teil der Oberfläche der Purkinjezellen ist durch eine Gliazellenlamelle gegen die Umgebung abgedichtet. Die absteigenden Äste der Korbzellenaxonen, die durch einen ungewöhnlichen Reichtum an Neurofilamenten ausgezeichnet sind, haben synaptische Kontakte lediglich mit dem axonalen Pol des Purkinje-Zellkörpers und mit dem gewöhnlich als Initialsegment des Axons betrachteten basalen Zellfortsatz. Als postsynaptische Formation sind im axonalen Pol des Zellkörpers, gegenüber Ansammlungen synaptischer Bläschen der Korbaxon-Endigungen, flache sackförmige Gebilde zu beobachten. Sie liegen parallel zu und unmittelbar unter der postsynaptischen Zellmembran. — Die Korbzell-Axonen-Endigungen verzweigen sich distal von ihren mit der Purkinjezelle und dessen Basalfortsatz eingegangenen synaptischen Kontakten in auswärts gerichtete fingerförmige Fortsätze die wenig synaptische Bläschen enthalten. Diese oft bürstenartig aus den Korbaxon-Enden hervorgehenden Fortsätze bilden in der äußeren Schicht des Korbes mit ähnlichen feinen Gliazellausläufern — der Bergmannschen Glia — ein lose verwobenes Geflecht. Die Gliazellfortsätze können auf Grund ihrer dichten Glykogenkörperchen leicht von den Korbaxon-Fortsätzen unterschieden werden. Dieses lose Geflecht enthält ungewöhnlich weite Interzellularräume die von einer leicht granulierten Matrix ausgefüllt sind. — Die funktionelle Bedeutung dieser Befunde wird im Lichte neuerer physiologischer Erkenntnisse über die hemmende Natur der Korbsynapse diskutiert

КОРЗИНЧАТЫЕ КЛЕТКИ ПУРКИНЬЕ: УЛЬТРАСТРУКТУРА ТОРМОЗЯЩЕГО СИНАПСА

Дается описание электронномикроскопической структуры корзинчатой клетки, окружающей клетку Пуркинье в мозжечке кошек. Преобладающая часть поверхности тела клетки Пуркинье отделена от смежных нервных элементов отростками глиозной клетки. Нисходящие ветви аксонов корзинчатой клетки, которые весьма богаты нейрофиламентами, образуют синаптический контакт лишь с базальной частью клетки Пуркинье и с начальной частью аксона. В этой начальной части аксона клетки Пуркинье обнаруживаются рибосомы и эндоплазматическая сеточка; следовательно, его еще нельзя рассматривать как аксон, правильнее было бы название «предаксон». Соответственно предсинаптическому накоплению пузырьков в постсинаптической части синапса, в клетке

появляются характерные постсинаптические структуры. Корзинчатые аксоны, после образования синапса с телом клетки Пуркинье или с предаксоном, разветвляются на весьма тонкие ветви, которые проникают во внешнюю часть волокнистой корзинчатой клетки и, следовательно, покрывают извне внутреннюю часть корзинчатой клетки, состоящую из более толстых волокон. Содержащие также синаптические пузырьки тонкие концевые ветви в большинстве случаев вступают и во внеклеточное пространство, которое, не заполнено клеточными отростками. Внеклеточное пространство содержит также тонкозернистый внеклеточный матрикс. В пространство местами могут вступить и глиозные отростки содержащие в большинстве случаев также глюкоген. Авторы обсуждают вышеизложенную ультраструктуру синапса и с точки зрения выдвинутой в последнее время гипотезы о тормозящей функции волокнистой корзинчатой клетки.

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Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója — Műszaki szerkesztő: Farkas Sándor

A kézirat nyomdába érkezett: 1965. IV. 16. — Terjedelem: 9,25 (A/5) ív, 56 ábra

65.60667 Akadémiai Nyomda, Budapest — Felelős vezető: Bernát György

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The *Acta Biologica* appear in parts of varying size, making up volumes.

Manuscripts should be addressed to:

Acta Biologica, Budapest IX., Tűzoltó u. 58.

Correspondence with the editors and publishers should be sent to the same address.

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Index: 26.005