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COMPARISON OF THE MORPHOLOGY OF STREPTOMYCIN-PRODUCING AND NONPRODUCING STRAINS OF STREPTOMYCES GRISEUS

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(Received October 5, 1962)

Synopsis

The life cycles of a streptomycin-producing strain (No. 52—1) and of a streptomycin-nonproducing strain (No. 45 H) of *Str. griseus* were compared by observing the morphological changes ensuing in submerged culture in a synthetic medium. Strain 45 H develops peculiar structures called "clumps" by us. The mycelia very soon differentiate into vegetative and reproductive forms. Latter need a very short time to split to spores. The life cycle of this strain is thus complete and short. In reproductive mycelia PAS-positive substance cumulates and, subsequently, shows characteristic changes in distribution related to spore formation. Strain 52—1 fails to form clumps. Its life cycle is incomplete and its development is slow. Significant quantity of PAS-positive substance appears in certain filaments, but — as far as its distribution is concerned — changes resembling those observable in the reproductive mycelia of strain 45 H do not appear here. The other streptomycin-nonproducing strains tested so far also form clumps, and their life cycle is also rapid and complete.

Introduction

SZABÓ, BARABÁS and VÁLYI-NAGY [16] studied the physiology and macromorphology of streptomycin-producing and streptomycin-nonproducing strains of *Str. griseus* in various solid and liquid media. The growth curves of the micro-organisms cultivated in submerged culture suggested that the life cycle of streptomycin-nonproducing strains was peculiarly short.

Closer analysis of the life cycles needed studies of the fine morphology of the strains. The morphology and histochemistry of *Streptomyces* strains were studied by numerous investigators [3, 4, 5, 6, 9, 10, 13, 14]. However, the results obtained were too general to answer the question raised by us. Finer and more specific markers were needed to distinguish streptomycin-producing strains from those producing no streptomycin. First of all we tried to reveal the dynamics of the histochemical changes and compare these results with those obtained by other members of our working group using different methods.

In order to arrive at correct conclusions, we had to study the problems of life cycle in general, too.

The present study makes a part of investigations aimed at elucidation of the significance of streptomycin in life processes of streptomycin-producing strains.

Materials and methods

Strains of Str. griseus. The streptomycin-producing strain No. 52—1 and its non-producing mutant No. 45 H [16] were studied. The micromorphological pictures were compared from time to time with those shown by other streptomycin-nonproducing strains (No. 187 and No. 176). In order to investigate life cycle, samples were taken from submerged culture. Shaken cultures growing in 100 ml culture medium in 500 ml Erlenmeyer flasks were used.

The culture medium contained 20.0 g glucose, 5.0 g asparagine, 2.0 g NH_4NO_3, 1.2 g KH_2PO_4, 2.9 g K_2HPO_4, 4.0 g sodium hexametaphosphate, 4.0 g $CaCO_3$, 1.0 g NaCl, 1.0 g $MgSO_4 \cdot 7\ H_2O$, 0.0064 g $CuSO_4 \cdot 5\ H_2O$, 0.0011 g $FeSO_4 \cdot 7\ H_2O$, 0.0015 g $MnCl_2 \cdot 4\ H_2O$, 0.0020 g $ZnSO_4 \cdot 7\ H_2O$ in 1000 ml distilled water; pH was adjusted to 7.4. To dissolve the constituents except glucose the mixture was placed in streaming steam for 15 minutes. Then glucose was added and the medium was sterilized by filtering through the Jena-Glass filter G-5.

Inoculation. Streptomyces cultures on soy agar slants [16] were shaken with glass beads to obtain spores. These were suspended in physiological saline and filtered through Schleicher-Schüll filter-paper 2043 b having been soaked in 0.67 per cent collodion to remove most of the mycelium fragments. (Spores pass through this filter, mycelial fragments are held back and the few which pass did not disturb our morphological investigations.) Culture medium was inoculated with the filtered spore suspension.

Samples for morphological examination were taken at 4, 8, 12, 18, 24, 36, 48, 72, 96, 120, 168, and 264 hours after inoculation. Location of the filaments and their relation to one another were examined by the phasecontrast microscope in fresh preparations. For histochemical purposes and for staining the samples were centrifuged, washed with saline, and after another centrifugation, suspended in distilled water and smeared on slides. The preparations were dried at room temperature. (This method was chosen because we had failed to observe any structural difference between fixed and unfixed mycelia.) For histochemical reactions needing fixation the preparations were fixed in a solution of 4% formaldehyde.

Staining for cell wall. Several procedures including GUTTSTEIN's [7, 8] tannic acid-crystal violet and dilute carbolfuchsin methods and the phosphomolybdenic-acid method of BISSET and HALE [2] were tried. These methods gave satisfactory results, particularly in thick mycelia. In most of the filaments, however, either the intensity of staining was insufficient or the plasma was also stained. For this reason a combined procedure was developed; unfixed preparations were treated with 1.5 per cent phosphomolybdenic acid for 5 minutes, washed three times with distilled water, and placed for ten minutes in a solution containing 20 per cent tannic acid and 4 per cent formalin. The preparations were then washed in three changes of distilled water for at least 10 minutes, stained with 1 : 5-diluted carbolfuchsin for 10 minutes, washed with tap water until drippings were colourless. This method of wall stain provides satisfactory pictures of a considerable number of mycelia, yet it is not suitable in every case. Prolongation of treatments with any of the solutions failed to improve or spoil the quality of preparation, whereas insufficient washing was greatly confusing.

Methylene blue stain. Unfixed preparations were kept in Loeffler's methylene blue solution for 1 hour, washed with tap water until no colour was seen in drippings and rinsed with distilled water.

Feulgen reaction. Formalin-fixed preparations were hydrolyzed in *N* HCl for 10 minutes and treated with Schiff's reagent for 1 hour [1].

Periodic-acid-Schiff (PAS) reaction. Formalin-fixed smears were treated according to MCMANUS and HOTCHKISS [1].

Results

Morphology of spores in the inoculum

The spores of strain No. 45 H are rounded and variable in size (Fig. 33). The smaller spores are approximately as large as the spores of strain No. 52-1, the larger ones are several times larger. The picture is unusually heterogeneous as regards form of spores, intensity of stain, metachromatism and orthochromatism. Observable amounts of PAS-positive substance are present, mainly in the wall of spores (Fig. 3).

The spores of strain No. 52-1 are cylindrical, relatively homogeneous in size and shape (Fig. 15). They are stained orthochromatically by methylene blue. Here, too, PAS-positive substance is demonstrable in the spore wall.

Life cycle

Before germination the spores of strain No. 52-1, too, round off. The spores of both strains begin to germinate as soon as after 4 hours of incubation. By 8—12 hours the majority of spores show germination. The mycelia growing out of the spores are thick, their walls are hardly detectable (Fig. 16). They are filled by a homogeneous basophilic substance showing orthochromatism with methylene blue. The PAS-positive substance is pale and diffuse (Figs. 23 and 34). Immediately after germination the two strains cannot be distinguished from each other. The mycelia of both strains show some ramification (Figs. 4, 5, 23).

Cultures of 12 *to* 18 *hours.* In this period cultures grow most rapidly. The mycelia grow in length while lose in thickness. Those of strain No. 52-1 show an evenly loose network (Fig. 17), whereas the network formed by strain No. 45 H, though consisting of similar thin filaments, develops very compact textures in certain foci. In this period the hyphae can still be followed in the texture; in the fine structure of the hyphae of the two strains no differences can be demonstrated (Figs. 24 and 35). The cores of mycelia show intensive orthochromatic staining by methylene blue.

Cultures of 24 *hours.* The network of strain No. 52-1 develops somewhat denser. There is no considerable difference between individual filaments (Fig. 18). The homogeneous, basophilic substance stainable with methylene blue splits into rods inside filaments. The PAS-reaction appears pale and shows diffuse colouration (Fig. 24).

Nearly all mycelia of strain No. 45 H cumulate in characteristic foci called "clumps" by us (Fig. 2). Low magnification shows the clump to be spherical with smooth surface or provided with shorter or longer mycelia forming "beard" on the surface. The spherical body itself is built up of con-

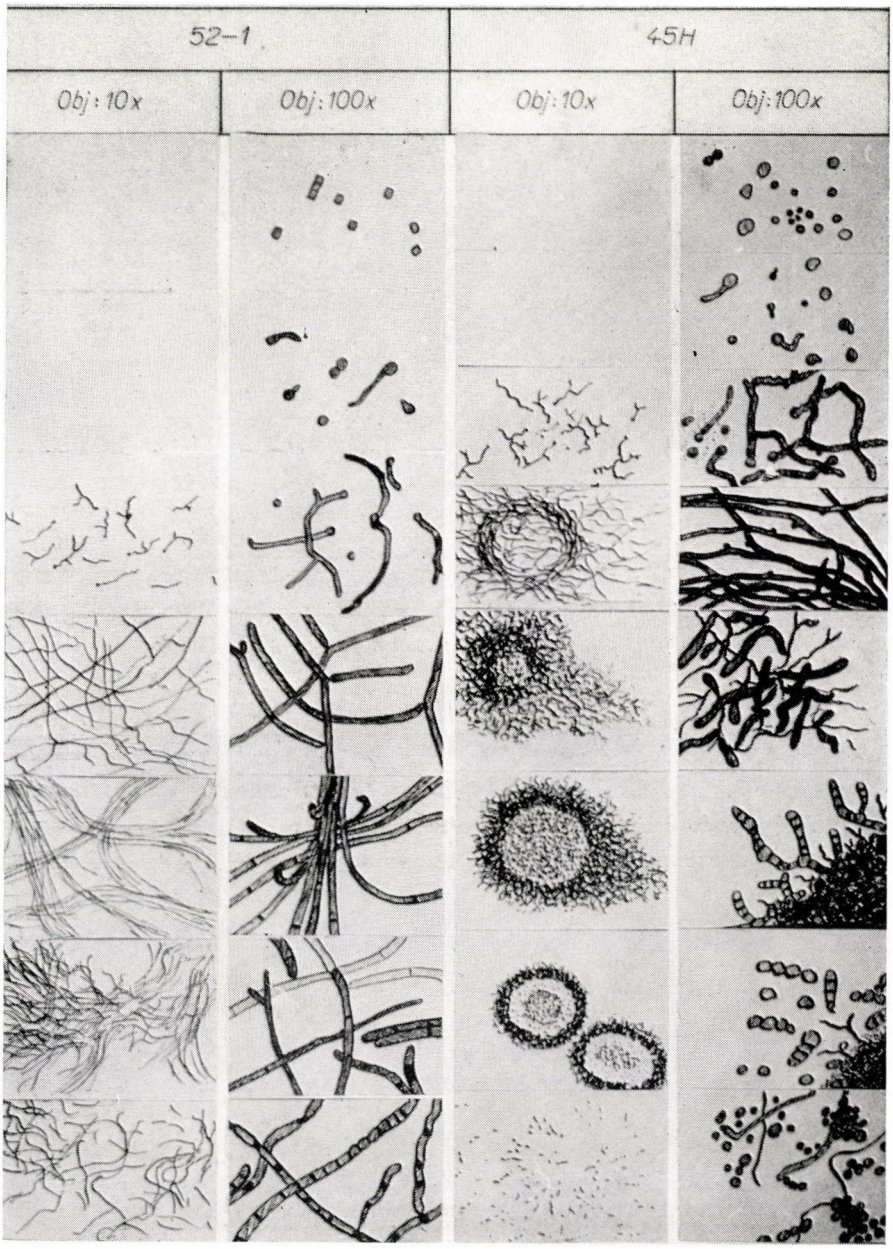
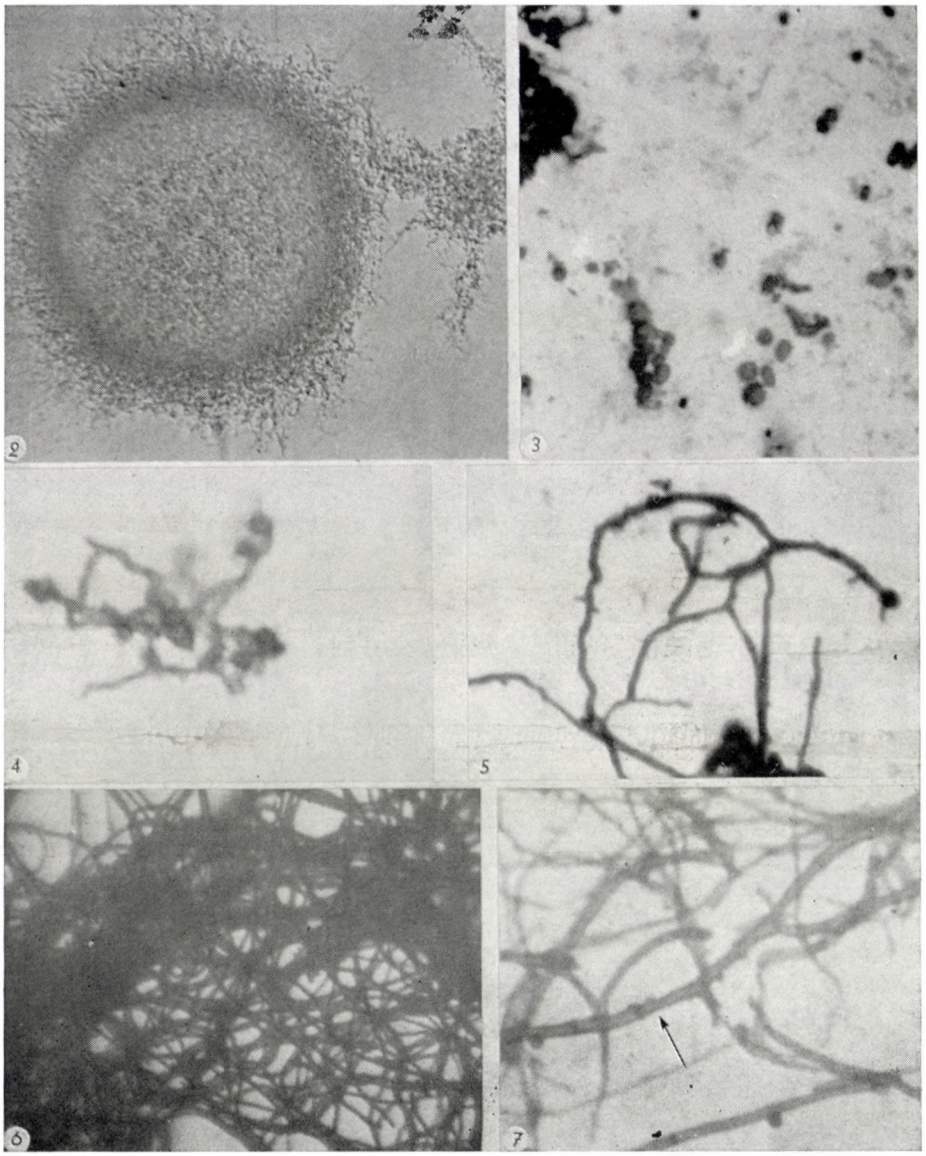


Fig. 1. Scheme of the development of the two strains. Forms of strain No. 52—1 presented in the last pictures of the two left columns appear only in soy-bean medium



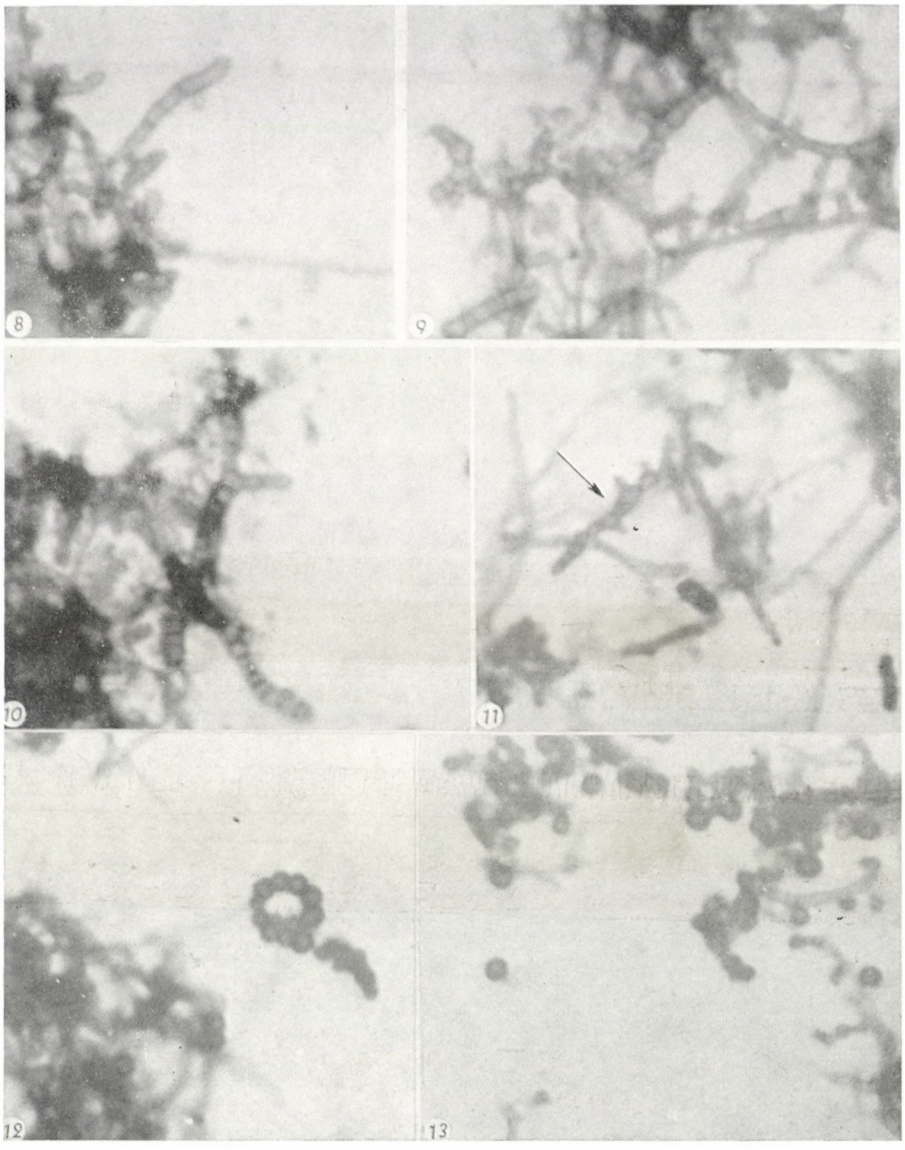
Figs. 2 to 13. Development of strain No. 45 H in synthetic medium. Wall stain. Magnification ×2000 if otherwise not stated. *Fig. 2.* Typical clump from 36-hour culture. Native preparation, ×120. *Fig. 3.* Spores suspended in saline and filtered through collodion-soaked filter paper. *Fig. 4.* Eight-hour culture. *Fig. 5.* Twelve-hour culture. *Fig. 6.* Twenty-four-hour culture. Mycelium network of a clump showing a compact marginal area (on the upper part of the picture) and a loose central one. Wall stain, ×1600. *Fig. 7.* Culture of 36-hours. Part of a clump crushed by pressing

centric zones. The outer (cortical) zone is denser than the intermediary one. The centre of the clump is denser again. In younger clumps this characteristic structure is not visible. First of all, hyphae cumulate showing a longer "beard" which later becomes shorter and forms the outer dense zone (24 hours). This is followed by the rarefied mid-zone and at last by the dense center. Examination of the clumps crushed by pressing by high magnification showes the clumps as consisting of mycelia (Fig. 6).

Subsequently, parallel with the development of the clump, the mycelia show highly progressive changes; as a result of these, extremely thick and thin mycelia develop (Fig. 7). The thick mycelia have proved to be branches of the thin ones. The thick mycelia show very pronounced wall staining. The cytoplasm of the mycelia being 1—1.5 μ in width is very intensely stained and shows orthochromatism with methylene blue. Initially the basophilic material filling the mycelia is homogeneous, only few fissures divide it into long rods. The thickness of the other filaments is variable, and only the thickest ones approximate 0.6 μ in thickness. Even these contain relatively much basophilic substance in long rods separated from one another by intervals, which are longer than those in the thick mycelia. The basophilic mass is stained with methylene blue orthochromatically. In the gaps light-violet-coloured material is visible. The PAS-stained thin mycelia show pale, diffuse colouration, while in the thick mycelia PAS-positive segments showing intense colour are observable (Fig. 37). This characteristic differentiation process was observed in the 48-hour cultures in every series of experiment.

Cultures of 48 *to* 60 *hours.* In thick mycelia of strain No. 45 H cross walls appear about as far from one another as wide the mycelium is (Figs. 8 to 10). Both the size of mycelia and the intervals between cross walls are very variable and irregular even within individual thick hypha. Accordingly parts greatly variable in shape and size are demarcated from one another within the mycelia. The cross walls are thicker and more pronounced than the side walls. When stained with methylene blue, the orthochromatic, intensely staining core of the mycelia appears to be divided into angular pieces and shows a picture corresponding to the negative of the preparation, stained for cell wall. The basophilic character of the thin mycelia weakens, the core of the mycelia appears light violet in colour. The PAS reaction gives pale, diffuse colouration in the filaments. In thick filaments the walls give an intensive PAS reaction (Figs. 36 to 38). The distribution of PAS-positive substance in individual thick mycelia is variable. As a rule, the PAS-positive segments of the cross walls are very pronounced and wider than the cross walls stained by ordinary cell wall staining.

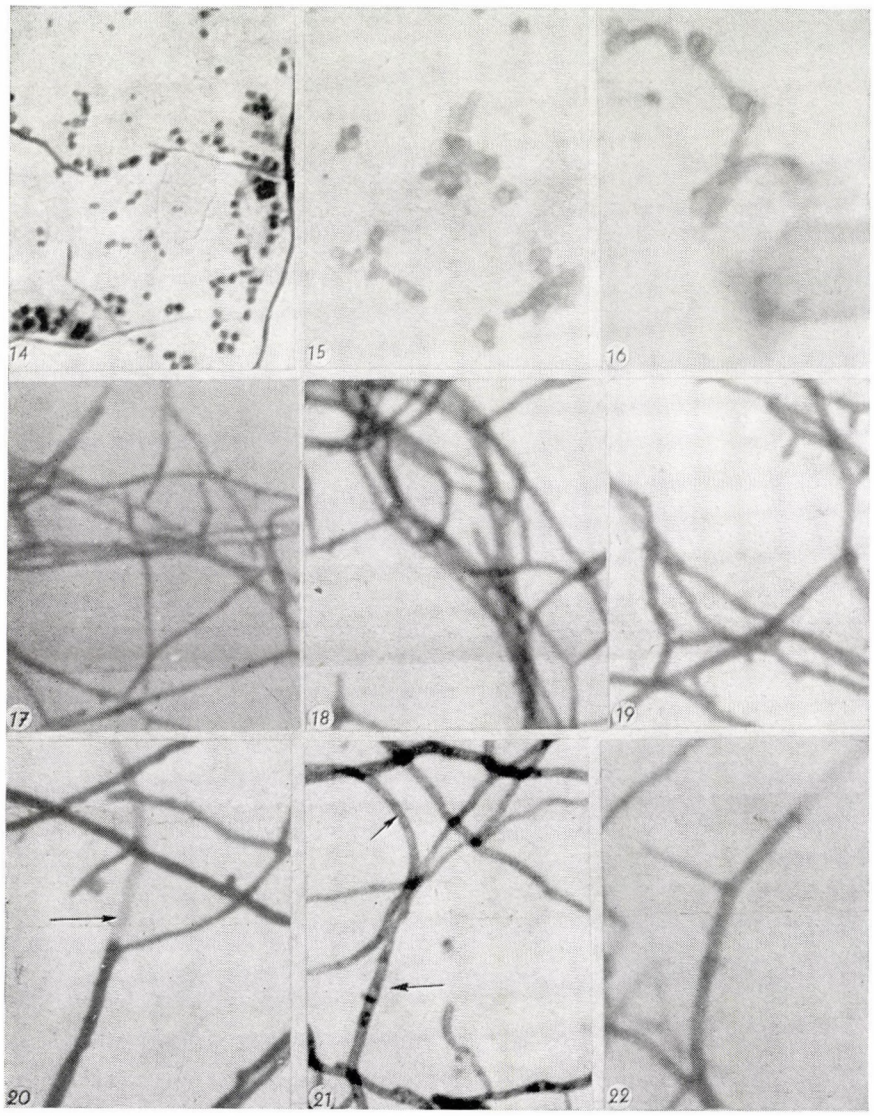
The cultures of strain No. 52-1 show little change in this period (Fig. 19). In smears stained with methylene blue rod-shaped, orthochromatic, basophilic substance and, beside the pale, violet-coloured core oval metachromatic



Figs. 8 to 10. Culture of 48 hours. Marginal parts of clumps. *Fig. 11.* Culture of 72 hours. Solitary reproductive mycelium fragment with rounded spores. *Fig. 13.* Culture of 168 hours

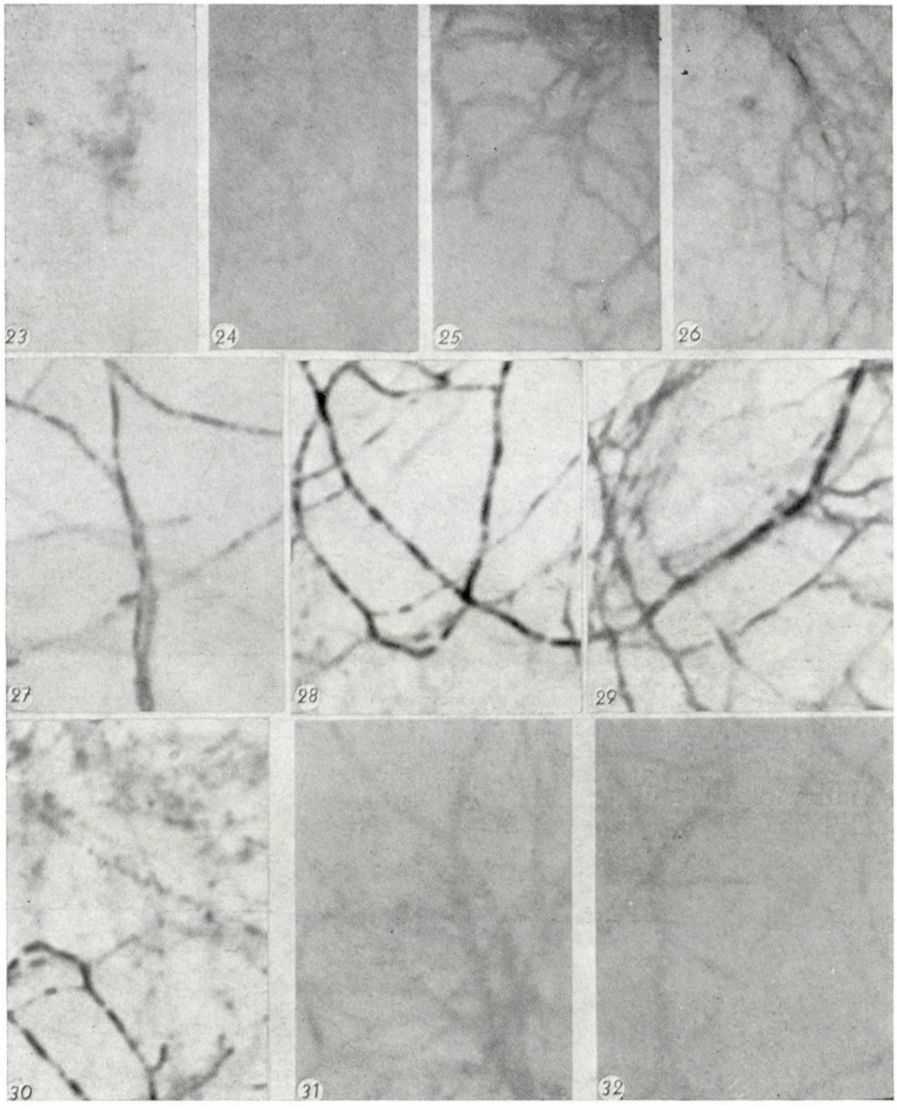
bodies appear. These are large, fill the whole width of mycelium and seem to bulge it. (Staining for wall does not show any bulge. This appears only when compared to the substance stainable by methylene blue.)

PAS reaction shows a regular series of rod-shaped, PAS-positive formations in certain mycelia; the other mycelia appear pale or coloured diffusely (Figs. 25 and 26).



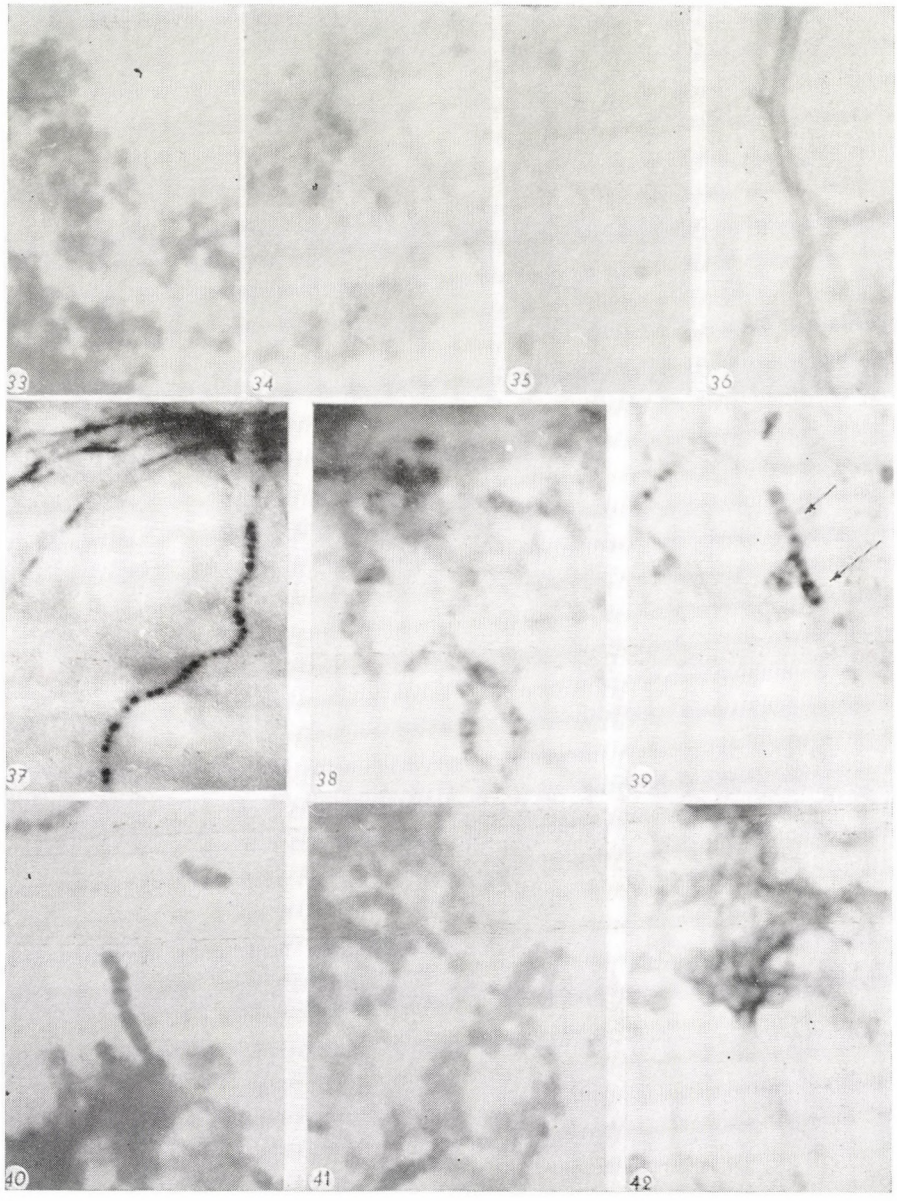
Figs. 14 to 22. Development of strain No. 52—1 in synthetic medium Magnification ×2000, if otherwise not stated. *Fig. 14.* Spores from slant soy-agar; crushed by pressing in dry state. ×1200. *Fig. 15.* Spores suspended in saline and filtered. ×1600. *Fig. 16.* Culture of 8 hours. *Fig. 17.* Culture of 18 hours. *Fig. 18.* Culture of 24 hours. *Fig. 19.* Culture of 48 hours. *Fig. 20.* Nine-day-old culture. The arrow shows a leached segment of mycelium. *Fig. 21.* Culture of 96 hours in filtered soy-bean medium. The arrows point to septate mycelia. *Fig. 22.* Eight-day old culture in filtered soy-bean medium

Cultures of 72 *and* 80 *hours.* The segments of the mycelia of strain No. 45 H, after having been separated from one another by thick walls, round off while still remain connected with each other, thus showing formations resembl-



Figs. 23 to 32. Strain No. 52—1. Changes in the distribution of the PAS-positive polysaccha-. rides. Synthetic medium. PAS-reaction. Magnification ×2000, if otherwise not stated *Fig. 23.* Culture of 8 hours. *Fig. 24.* Culture of 24 hours. *Fig. 25.* Culture of 36 hours. *Fig. 26.* Culture of 48 hours. ×1400. *Fig. 27.* Culture of 72 hours. *Figs. 28, 29 and 30.* Cultures of 96 hours. *Fig. 31.* Culture of 120 hours. *Fig. 32.* Culture of 168 hours

ing strings of beads (Figs. 11 and 12). The thickness of the wall of developed spores appears to be uniform. Spore chains consist of spores rather variable in size and shape. When stained with methylene blue, the spores appear to be filled by a basophilic substance. The Feulgen test shows a small, round, Feulgen-positive nucleoid in the centre of each spore. The nucleoid is much



Figs. 33 to 42. Strain No. 45 H. Changes in the distribution of the PAS-positive polysacchar es. Synthetic medium. PAS reaction. Magnification ×2000 if otherwise not stated. *Fig. 33.* Spores suspended in saline and filtered. *Fig. 34.* Culture of 8 hours. *Fig. 35.* Culture of 18 hours. *Fig. 36.* Culture of 48 hours. PAS-positive polysaccharides in side walls. *Fig. 37.* Culture of 48 hours. Spherical and disc-shaped polysaccharide bodies corresponding to the subsequent cross walls. Above: rod-shaped formations reminding of similar bodies in cultures of strain No. 52—1. ×1600. *Fig. 38.* Culture of 48 hours. Increased amounts of PAS-positive polysaccharides in both the cross and side walls. *Fig. 39.* Culture of 72 hours. Arrows point to bracket-like distribution of PAS-positive substances. *Fig. 40.* Culture of 96 hours. PAS-positive polysaccharides surround the spores of spore chains evenly. *Fig. 41.* Culture of 168 hours. *Fig. 42.* Culture of 264 hours, reminding of the initial picture

smaller in diameter than the spore. In spores different in size the nucleoids seem to be of equal size, larger ones are uncommon. The intensity of staining of spores by methylene blue is variable, and in some spore chains spores showing metachromatism and those showing orthochromatism succeed each other without any rule. Inside of the spores the basophilic substance appears to be homogeneous in distribution and colour when examined under the light-microscope. Methylene blue stain of spore chains provides a picture resembling the negative of the preparation stained for wall. PAS reaction gives intensive colouration (Figs. 39 and 40). It should be noted that the PAS-positive substance does not cover the cell wall evenly; the PAS-positive zone is relatively wide at the cross wall and is gradually thinning towards the middle of the segment and so, like a bracket, encloses the spore (Fig. 39). Later, when the spores have been separated from each other, this distribution of the PAS-positive substance changes and the substance surrounds the spore evenly. Spore chains split into shorter chains or solitary spores. The spores tend to aggregate (Figs. 13, 41, and 42). The vegetative mycelia are stained less and less intensely until they are fully dissolved. Some resistant, middle-thick, well-stainable filaments may remain unchanged over 15 days or more (Fig. 13).

Strain No. 52-1 does not show much change after 48 hours (Figs. 19 to 22, and 27 to 32). Cross walls are placed far from one another, the filaments are long and relatively thin. Some filaments and certain segments of some filaments are first leached, then dissolved (Fig. 20). The basophilic character gradually weakens and more and more filaments appear being autolyzed. After 10—15 days intact fragments of filaments are visible among shadow-like ones. The Feulgen reaction fails to show variability in the distribution of DNA among individual filaments.

DNA appears in the form of rods or spherical formations, in agreement with previous data [10, 15]. Appearance of cross walls is very uncommon. Sporulation could not be detected in deep cultures having grown in the synthetic medium employed, not even after 10—20 days of incubation. Submerged culture in filtered soy-bean medium produced mycelia with cross walls placed nearer to each other, but even these were greatly different from the reproductive mycelia of strain No. 45 H; their thickness was uniform, the segments between cross walls were approximately identical in size and square-like in shape (Figs. 21 and 22). Cross walls were not formed at the ends of mycelia and the segment divided by cross-walls continues in both directions in mycelium-segments in which the cross-walls are fairly remote from each other.

Discussion

The morphological and histochemical characteristics of two strains, *viz.* the streptomycin-producing strain No. 52-1 and its streptomycin-nonproducing

variant No. 45 H, were compared to each other in shaken cultures grown in synthetic medium. Differentiating markers of their life cycles were sought.

Certain morphological and histochemical events are common for every *Streptomyces* strain; other markers are characteristics of certain strains and appear in those consistently. A third group of histochemical markers need special conditions to appear, even in strains capable of developing such features. In order to elucidate the life cycles, one should analyze the observed changes accordingly. However, literature does not provide sufficient basis and our own experiments are too limited to analyze the data with complete security.

The life cycle of strain No. 45 H is complete in deep culture growing either in soy-bean medium or in a synthetic one; a great number of spores is formed by the end of the cycle. The period of sporulation follows the way corresponding to that designed by a—b—c—d—d_1—d_2 in the scheme of PÉNAU and co-workers (Fig. 43) [13]. The vegetative period of the life cycle is rather short. Walls tending to separate spores from one another appear as early as between 48—60 hours even in the synthetic medium. These walls indicate a progressed state of the reproductive phase. The reproductive phase keeps on long; new reproductive mycelia are still formed on the fourth day of incubation. The strain develops characteristic aggregation of mycelia (called clumps by us). The observations obtained so far are insufficient to reveal the way of development of these clumps and their role played in the short life cycle. (Other streptomycin-nonproducing strains, *viz.* No. 176 and No. 187, also produce clumps and, also, their life cycles are short. Parallelism or, at least, unilateral correlation was observed between these two phenomena: clump-formation consistently indicated short life cycle.) (Fig. 1).

During the life cycle peculiar changes in the distribution of polysaccharides were observed in reproductive mycelia, parallel with the course of sporulation. Although our experimental data are insufficient to draw final conclusion, these changes do not seem to be characteristic of the strain under study, rather of sporulation in general.

The most pronounced micromorphological characteristics of the strain are heterogeneity of spores in shape, size, and staining. The variability in staining does not mean that some spores are sterile, others include nuclei; nor does it mean detectable differences in the quantity of DNA. The factors responsible for heterogeneity in staining may be located either in the plasma or in the walls.

The life cycle of strain No. 52-1 is incomplete in deep cultures. Neither spores are formed, nor the polysaccharides are re-arranged. The morphological picture remains unchanged for a long period (Fig. 1).

The synthetic medium used by us proved to be favourable; both strains grew well in it and the morphological characteristics of strains were much more

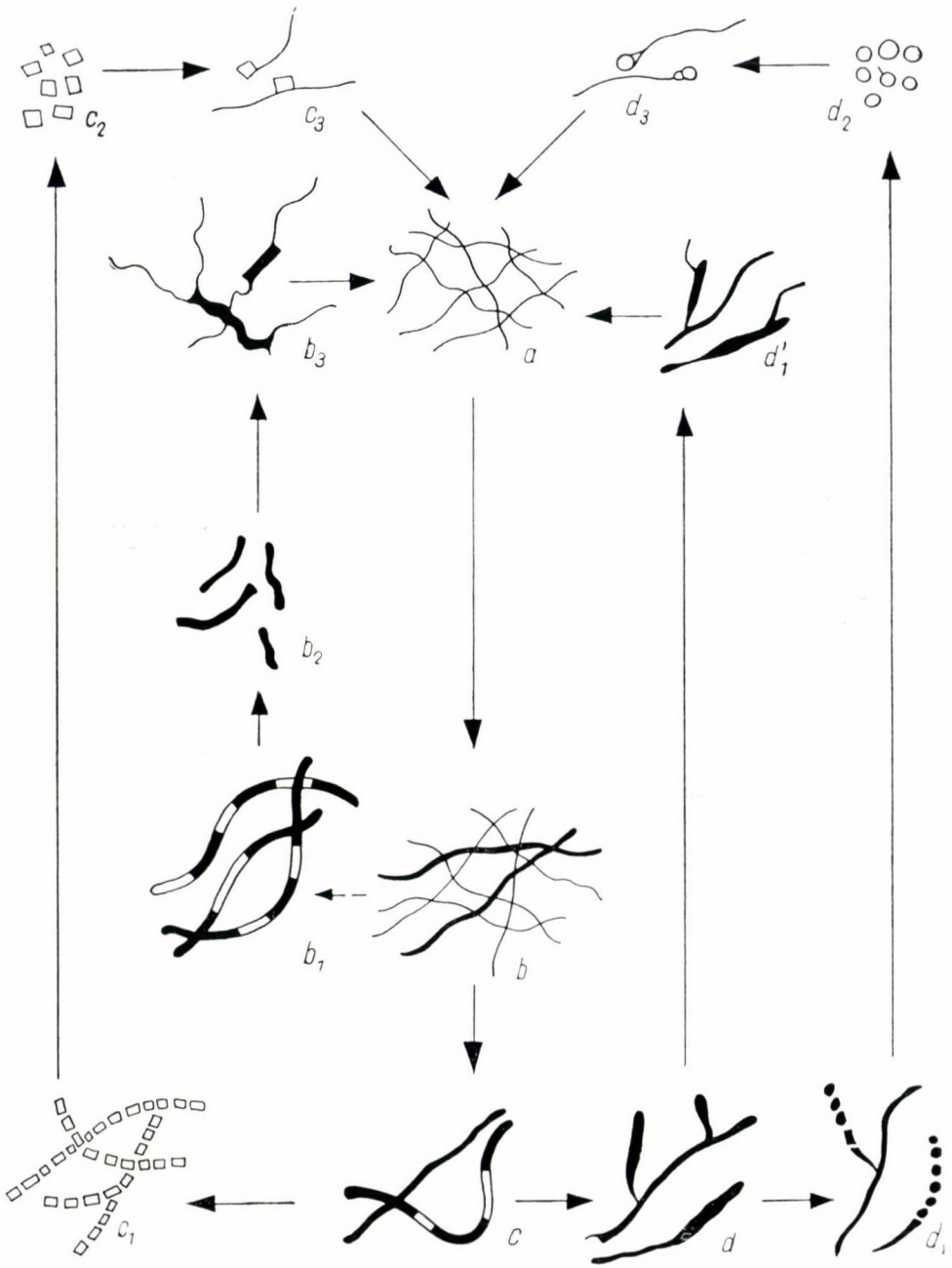


Fig. 43. PÉNAU's scheme [13]

pronounced in this than in the soy-bean medium; phenomena appearing as uncertain tendencies in the latter, developed to valuable morphological markers in the former.

Further conclusions need thorough study of the life cycles of several distinct strains and of the conditions of clump formation.

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VERGLEICHENDE MORPHOLOGISCHE UNTERSUCHUNG VON STREPTOMYZIN PRODUZIERENDEN UND NICHT PRODUZIERENDEN STREPTOMYCES GRISEUS-STÄMMEN

Auf Grund der Veränderungen des morphologischen Bildes wurden die Lebenszyklen eines Streptomyzin produzierenden (Nr. 52—1) und eines Streptomyzin nicht produzierenden *Streptomyces griseus*-Stammes (Nr. 45-H) bei Tiefenfermentation auf synthetischen Nährboden untersucht.

Bei dem Stamm Nr. 45-H entstehen eigenartige von den Verfassern als Knötchen bezeichnete Gebilde. Die Myzelien differenzierten sich sehr bald zu vegetativen und reproduktiven Formen. Die reproduktiven Myzelien zerfielen in äusserst kurzer Zeit zu Sporen. Der Lebenszyklus ist also vollständig und geht schnell vor sich. In den reproduktiven Myzelien ist die Menge der PAS-positiven Stoffe grösser, und in ihrer Verteilung kann man im Zusammenhang mit der Sporenbildung charakteristische Veränderungen beobachten.

Beim Stamm Nr. 52—1 entstehen keine Knötchen, der Lebenszyklus ist nicht vollständig und die Entwicklung verläuft langsam. Auch bei diesem Stamm lässt sich in einzelnen Myzelienfäden eine erhebliche Menge von PAS-positivem Stoff nachweisen, in dessen Verteilung jedoch die bei dem Stamm Nr. 45-H beobachteten Veränderungen nicht erfolgen.

Die bisher untersuchten, kein Streptomyzin erzeugenden *Streptomyces griseus*-Stämme zeigen gleichfalls ein noduläres Wachstum, ihr Lebenszyklus verläuft rasch und vollständig.

СРАВНИТЕЛЬНОЕ МОРФОЛОГИЧЕСКОЕ ИССЛЕДОВАНИЕ ШТАММОВ STREPTOMYCES GRISEUS, ПРОИЗВОДЯЩИХ И НЕ ПРОИЗВОДЯЩИХ СТРЕПТОМИЦИН

Авторы на синтетической питательной среде при глубокой ферментации на основании изменения морфологической картины сравнивали жизненный цикл штамма *Streptomyces griseus* (№ 52—1), производящего стрептомицин и штамма (№ 45—Н), не производящего стрептомицин.

У штамма № 45—Н формируются своеобразные образования, которые авторы назвали бугорками. Мицелии весьма быстро дифференцируются в вегетативные и воспроизводительные формы. Воспроизводительные мицелии в течение весьма короткого времени распадаются на споры. Значит, жизненный цикл полный и осуществляется быстро. В воспроизводительных мицелях количество PAS-положительных веществ больше и в распределении этих PAS-положительных веществ наблюдаются характерные изменения в связи с процессом спорообразования.

У штамма № 52—1 бугорки не образуются. Жизненный цикл неполный, развитие медленное. В отдельных нитях у этого штамма также появляется более значительное количество PAS-положительного вещества, но в его распределении не происходят тех характерных изменений, которые наблюдаются у штамма № 45—Н.

Исследованные до сих пор штаммы *Streptomyces griseus*, не синтетизирующие стрептомицин, также образуют бугорки, их жизненный цикл быстрый и полный.

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CHANGES OF NUCLEIC ACID CONTENT IN GASTROCNEMIUS MUSCLES OF PIGEONS AND TURTLES AFTER DENERVATION AND TENOTOMY

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Synopsis

The change in nucleic acid content has been studied in denervated and tenotomized gastrocnemius muscles of *Columba domestica* and *Emys orbicularis* on the 5th, 10th, 15th and 20th postoperative days. In the denervated pigeon muscle the quantity of DNA related to 100 mg of dry tissue and defined in relation to the contralateral muscle increased, and that of RNA decreased. In the denervated turtle muscle the RNA content decreased, while the DNA content did not show any appreciable change. After tenotomy the change in nucleic acid content was less than after neurotomy. Denervation was followed by a greater change in nucleic acid content in the phylogenetically higher species.

Introduction

In our former investigations [3] it was established that the interruption of peripheral innervation was followed by a significant change of nucleic acid content in the striated muscles of mammals: in the denervated gastrocnemius muscle of the rat the desoxyribonucleic acid (DNA) content showed an increase, while the quantity of ribonucleic acid (RNA) decreased.

The data in the literature of this field are divergent. Mandel and Jacob [5] found the DNA content unchanged on the 30—40th postoperative days while the quantity of RNA had decreased to 30—60 per cent. Hearn reported similar results [2]. Schmidt and Schlief [6] observed an increase of both nucleic acids in atrophied muscles; Gutmann and Žak [1] reported on an increase in the absolute quantity of RNA.

In the present paper we have studied the differences between the various groups of Amniotes in the regulatory role played by the nervous system in the metabolism of nucleic acids, and also the problem of how far the metabolism of DNA and RNA is influenced by the interruption of neural control of the striated muscle tissue in the investigated species.

Methods

Adult pigeons *(Columba domestica)* and turtles *(Emys orbicularis)* of either sex were used. Under ether anaesthesia and aseptic conditions a 10 mm part of the sciatic nerve was excised unilaterally, to achive total denervation of the gastrocnemius muscle. The contralateral muscle served as control.

To determine in what measure the nucleic acid content of the muscle was influenced by inactivity alone in another group of animals monolateral Achilles tenotomy was performed.

The nucleic acid content of gastrocnemius muscles was determined by means of U. V. spectrophotometry using the method of TSANEV and MARKOV [8] on the 5th, 10th, 15th and the 20th postoperative days. In the preparation of tissue powder we modified the original method of these authors, in the first place by applying a more effective removal of the lipids. According to our observations it was thus possible to increase the extractibility of DNA from the muscle tissue, and the results of U. V. absorption measurements were more in agreement with those of the Schmidt—Tannhauser method, because of the complete removal of the lipid-P.

The method for determining nucleic acids was as follows:

Fresh tissue :

200—250 mg homogenized in 5 vol. 5% ice-cold trichloroacetic acid (TCA).
Stirring in a centrifuge tube (90×18 mm) at 0—3° C for 20 min.
Centrifuged at 1050×g, 0—3° C, 10 min.
Residue is dissolved in 5 vol. ice-cold 10% TCA. Stirring. Centrifugation.
Repetition of the extraction until supernatant is free of phosphorus.

Tissue residue : (proteins, lipids, nucleic acids)

Extract : acid-soluble nucleotides, sugar phosphates, glycogen, etc.

Lipid extraction:
10 ml ice-cold ethanol (66%), stirring for 15 min.
10 ml ethanol (96%), stirring at room temperature, 15 min.
10 ml chloroform-96% ethanol (3 : 1), in water bath at 55° C, 30 min.
10 ml ether, 40° C, 20 min.
10 ml ether, 40° C, 10 min. Centrifuged.

Sediment : proteins, nucleic acids

Extract : lipids

Drying in thermostat at 37° C for 24 hours.

Measuring of tissue powder (20 mg) and digestion in KOH (1 ml 0.5 *N* KOH per 100 mg fresh tissue) 37° C, 18 hours.

Cooling. Acidification to pH 3. Centrifugation.

Precipitate : DNA and proteins

Extraction of DNA
by 5 ml *N* $HClO_4$ at 80° C for 30 min. Centrifugation. The extraction is repeated once more.

Measurement of supernatant DNA content in 15 ml vol. at 268 and 284 mu.

Supernatant :
RNA as mononucleotides

The *measurement of* combined supernatant *RNA content* in 25 ml vol. at 260 and 286 mu.

U. V. measurements were done by means of a Beckman DU Spectrophotometer. As standards desoxyribonucleic acid sodium salt (made from calf thymus, L. Light and Co. Ltd. Colnbrook, England), and ribonucleic acid sodium salt (Fluka AG., Switzerland) were used.

Significance was computed according to STUDENT's test [7].

Results

The weight changes of denervated and tenotomized muscles

After denervation and tenotomy the weights of gastrocnemii of *Columba domestica* decreased as compared with the contralateral controls. In the birds tenotomy caused a more marked atrophy than denervation did. Twenty days after denervation the weight of the pigeon muscles decreased on an average, by 7.66 per cent and 20 days after tenotomy, by 41.55 per cent.

In the denervated muscles of *Emys orbicularis* oedematous changes could be observed, resulting in increased weight of muscles. The maximum increase was reached on the 10th postoperative day (13.5%), and then a return to normal started. There was no significant change of muscle weight in the tenotomized turtles.

The change in the nucleic acid content

Depending on the species and the surgical interference the loss of weight of tenotomized and neurotomized muscles was different, which can be attributed, in the first place, to differences in water content. To exclude the non-uniform changes of water content, as a factor rendering comparison of the results more difficult, the nucleic acid content of the muscles was related to 100 mg dry tissue weight. In denervated pigeon muscles the quantity of DNA increased in relation to the contralaterals (Table 1). A significant rise could be demonstrated on the 10th day, while the maximum rise occurred on the 15th

Table 1

DNA content of denervated and normal gastrocnemius muscles of Columba domestica

Number of animals	Post-operative days	DNA–P content of normal muscles (N) (μg P/100 mg dry tissue)*	DNA–P content of denervated muscles (D) (μg P/100 mg dry tissue)*	Per cent difference between series N and D	Significance between series N and D
15	5	20.8±0.2	21.4±0.1	+ 2.9	> 0.7
15	10	20.4±0.3	23.1±0.1	+13.2	< 0.01
15	15	20.3±0.2	23.7±0.4	+16.7	< 0.001
15	20	21.0±0.2	24.5±0.2	+16.6	<0.001

* mean ± standard error of the mean

day. In tenotomized muscles a rise, low in degree, but significant could be observed only on the 15th day (Table 2).

The change in RNA content showed a decreasing tendency in denervation as well as in tenotomy (Tables 3 and 4).

The change of RNA content induced by tenotomy was of lesser measure than that observed in the case of denervation and reached a significant level only on the 15th day. In the denervated and tenotomized turtle muscles the DNA content did not show any significant change during the period of investigation (Tables 5 and 6). The quantity of RNA changed only in the dener-

Table 2

DNA content of tenotomized and normal gastrocnemius muscles of Columba domestica

Number of animals	Post-operative days	DNA–P content of normal muscles (N) (μg P/100 mg dry tissue)*	DNA–P content of tenotomized muscles (T) (μg P/100 mg dry tissue)*	Per cent difference between series N and T	Significance
15	5	20.3±0.4	20.6±0.1	+1.4	
15	10	19.9±0.3	20.8±0.3	+4.5	> 0.05
13	15	20.3±0.3	22.1±0.5	+8.8	< 0.01
15	20	20.7±0.1	21.3±0.1	+2.8	

* mean ± standard error of the mean

Table 3

RNA content of denervated and normal gastrocnemius muscles of Columba domestica

Number of animals	Post-operative days	RNA–P content of normal muscles (N) (μg P/100 mg dry tissue)*	RNA–P content of denervated muscles (D) (μg P/100 mg dry tissue)*	Per cent difference between series N and D	Significance
15	5	36.5±0.4	35.0±0.5	— 4.1	
15	10	35.8±0.3	29.4±0.5	—17.9	< 0.001
13	15	36.2±0.3	29.1±0.3	—19.7	< 0.001
15	20	36.2±0.2	31.3±0.5	—13.5	< 0.001

* mean ± standard error of the mean

Table 4

RNA content of tenotomized and normal gastrocnemius muscles of Columba domestica

Number of animals	Post-operative days	RNA–P content of normal muscles (N) (μg P/100 mg dry tissue)*	RNA–P content of tenotomized muscles (T) (μg P/100 mg dry tissue)*	Per cent difference between series N and T	Significance
15	5	36.6 $\pm$ 0.4	35.2 $\pm$ 0.3	— 3.8	
15	10	34.0 $\pm$ 0.4	35.3 $\pm$ 0.4	— 4.9	> 0.06
13	15	36.4 $\pm$ 0.3	32.4 $\pm$ 0.4	—10.9	< 0.01
15	20	35.8 $\pm$ 0.1	33.1 $\pm$ 0.5	— 7.7	< 0.03

* mean $\pm$ standard error of the mean

Table 5

DNA content of denervated and normal gastrocnemius muscles of Emys orbicularis

Number of animals	Post-operative days	DNA–P content of normal muscles (N) (μg P/100 mg dry tissue)*	DNA–P content of denervated muscles (D) (μg P/100 mg dry tissue)*	Per cent difference between series N and D	Significance
8	5	29.3 $\pm$ 0.1	29.3 $\pm$ 0.3	—	
5	10	28.9 $\pm$ 0.2	30.0 $\pm$ 0.1	+3.8	> 0.8
5	15	29.5 $\pm$ 0.1	30.4 $\pm$ 0.1	+3.1	> 0.8
5	20	29.2 $\pm$ 0.4	30.2 $\pm$ 0.3	3.4	> 0.7

* mean $\pm$ standard error of the mean

Table 6

DNA content of tenotomized and normal gastrocnemius muscles of Emys orbicularis

Number of animals	Post-operative days	DNA–P content of normal muscles (N) (μg P/100 mg dry tissue)*	DNA–P content of tenotomized muscles (T) (μg P/100 mg dry tissue)*	Per cent difference between series N and T	Significance
8	5	29.6 $\pm$ 0.6	29.8 $\pm$ 0.3	+0.6	
5	10	29.5 $\pm$ 0.2	30.3 $\pm$ 0.3	+2.7	
5	15	29.0 $\pm$ 0.4	29.9 $\pm$ 0.2	+3.1	> 0.5
5	20	29.5 $\pm$ 0.3	30.2 $\pm$ 0.3	+2.3	

* mean $\pm$ standard error of the mean

Table 7

RNA content of denervated and normal gastrocnemius muscles of Emys orbicularis

Number of animals	Post-operative days	RNA–P content of normal muscles (N) (μg P/100 mg dry tissue)*	RNA–P content of denervated muscles (D) (μg P/100 mg dry tissue)*	Per cent difference between series N and D	Significance
8	5	45.1±0.5	44.2±0.3	— 2.0	
5	10	44.7±0.2	40.9±0.6	— 8.2	< 0.03
5	15	45.5±0.4	40.6±0.5	—10.7	< 0.01
5	20	44.7±0.5	40.1±0.5	—10.3	< 0.02

* mean ± standard error of the mean

Table 8

RNA content of tenotomized and normal gastrocnemius muscles of Emys orbicularis

Number of animals	Post-operative days	RNA–P content of normal muscles (N) (μg P/100 mg dry tissue)*	RNA–P content of tenotomized muscles (T) (μg P/100 mg dry tissue)*	Per cent difference between series N and T	Significance
8	5	45.6±0.2	46.0±0.4	+0.8	
5	10	45.0±0.5	45.1±0.6	+0.2	
5	15	45.1±0.2	43.1±0.3	—4.4	
5	20	45.6±0.6	43.2±0.4	—5.3	> 0.6

* mean ± standard error of the mean

vated muscles, showing a decrease of 10.7 per cent and one of 10.3 per cent on the 15th and 20th days, respectively (Tables 7 and 8).

Discussion

These experiments have shown that denervation of striated muscles in the investigated species, and the state of inactivity produced by tenotomy are followed by changes in nucleic acid content which are similar to those seen in mammals. But the intervention influenced the DNA metabolism in a lesser degree. It can be established that after denervation the nucleic acid content showed a change of a higher degree in the phylogenetically higher species. This

is proved also by our observations made on Anamnia *(Rana esculenta)*. We failed to demonstrate any change of the nucleic acid content in the denervated muscles of these phylogenetically lower animals, even in the second postoperative month. One explanation of the difference in the change of nucleic acid content observed in different species, may be that the time course of muscle degeneration exhibits species variations as demonstrated by KNOWLTON [4]. In addition the difference in metabolic rate between the poikilothermic and homoiothermic animals must be born in mind.

In the case of bird muscles, the inactivity atrophy occurring within the investigation period was of a higher degree than the atrophy observed in the denervated muscles, but in spite of that the change of the nucleic acid content computed for 100 mg dry tissue weight was always higher in the denervated muscles than in the tenotomized ones. This observation also supports the existence of the trophic effect assumed by us.

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DIE ÄNDERUNG DES NUKLEINSÄUREGEHALTES IM MUSCULUS GASTROCNEMIUS DER TAUBE UND SCHILDKRÖTE NACH DENERVATION UND TENOTOMIE

Die Änderung des Nukleinsäuregehaltes im denervierten und tenotomierten Musculus gastrocnemius von *Columba domestica* und *Emys orbicularis* wurde am 5., 10. und 20. Tag nach dem operativen Eingriff studiert.

Im denervierten Taubenmuskel steigt die auf 100 mg Gewebstrockengewicht bezogene und mit dem Muskel der anderen Seite verglichene DNS-Menge, während die RNS Menge sinkt. Im denervierten Schildkrötenmuskel vermindert sich die RNS Menge, wobei der DNS Gehalt sich nicht signifikant verändert. Nach Tenotomie verändert sich der Nukleinsäuregehalt in geringerem Masse als im Falle der Neurotomie.

Bei den phylogenetisch entwickelteren Arten folgt auf einen Innervationsausfall eine bedeutendere Veränderung des Nukleinsäuregehaltes.

ИЗМЕНЕНИЕ СОДЕРЖАНИЯ НУКЛЕИНОВЫХ КИСЛОТ В ИКРОНОЖНЫХ МЫШЦАХ ГОЛУБЕЙ И ЧЕРЕПАХ ПОСЛЕ ДЕНЕРВАЦИИ И ТЕНОТОМИИ

Изучали изменение содержания нуклеиновых кислот в икроножных мышцах ***Columba domestica*** и ***Emys orbicularis*** после денервации и перерезки сухожилия на 5., 10., 15. и 20. день после операции.

Количество дезоксирибонуклеиновой кислоты в денервированной икроножной мышце голубя (на 100 мг. сухого веса ткани по отношению противоположной стороны) повышается, а количество рибонуклеиновой кислоты понижается. В венервированной мышце черепахи количество рибонуклеиновой кислоты понижается, а содержание дезоксирибонуклеиновой кислоты не меняется сигнификантно. Содержание нуклеиновых кислот менее выраженно меняется после перерезки сухожилия, чем после нейротомии. У филогенетически низших видов больше изменяется содержание нуклеиновых кислот при выпадении иннервации.

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SEARCH FOR ANTAGONISTIC ACTINOMYCETAE IN HUNGARIAN SOILS

III. VIABILITY OF STREPTOMYCES ISOLATES PRESERVED IN SOIL CULTURES

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Synopsis

The viability of *Streptomyces* isolates in various "soil cultures" was studied. Out of the materials applied to prepare soil cultures, soil samples obtained from the habitats proved to be most suitable for the preservation of isolates. Washed clay was also utilizable, but in washed, glowed and sterilized quartz sand the cultures soon perished. Organic matter (humus) content of the soil of the habitats does not influence the viability of isolates during storage in soil cultures. Isolates obtained from habitats with ionizing radiation (uranium soils) were less viable than those obtained from non-radiating habitats.

Isolates of soil microorganisms often have to be preserved under laboratory conditions until their antibiotic-producing capacity, or possible cytostatic or virucidal *etc.* effects, are determined. In the case of certain moulds and Actinomycetae considerable problems arise. The maintenance of several thousands of isolates in vegetative cultures, *i.e.* in continuous passages, is highly expensive and work-consuming while often not assuring the preservation of the original characteristics of the isolates.

In order to preserve their viability and morphological, physiological, cultural and biochemical characteristics fungi were successfully preserved and stored by lyophilizing their spores or vegetative forms [2, 3].

Another method, not less generally applied in the preservation of fungi is the preparation of "soil cultures". GREENE and FRED [1] were the first to apply this method to maintain preparatory cultures of fungi in the laboratory. They succeeded in preserving fungi in such cultures for a number of years. *Streptomyces* strains and isolates have also been maintained in some laboratories by the soil culture method, namely by drying dense spore suspensions in sterile clay. When screening for antagonistic effect *Streptomyces* strains and isolates VÁLYI-NAGY and co-workers often observed rapid decrease in the viability of spores preserved in washed clay, and this decrease was especially striking in the case of *Streptomyces* isolates that proved to be antagonistic.

Since no literary data were available on preservation of *Streptomyces* spores or parts of mycelia in dry soil cultures the present experiments have been initiated. We wanted (i) to reveal the influence of the soil on the preservation of isolates and (ii) to demonstrate the possible influence of the conditions prevalent at the habitat on the viability of *Streptomyces* isolates.

Materials and methods

Streptomyces isolates. The best spore-forming isolates obtained from various soils of Hungary in the years 1959 to 1960 and stored in our strain collection were tested.

The isolates were inoculated into soy bean medium and incubated in a thermostat at 27° C for 7 to 10 days. During this period abundant growth and spore formation were observed on the surface of the medium; the isolates formed a confluent sheet. Subsequently the colonies were suspended in two different fluids, *viz.* (i) in sterile saline (0.9 per cent sodium chloride) and (ii) in an extract of the own soil of the isolate. These extracts were prepared as follows. The soil was dried, pulverized, and suspended in two volumes of distilled water. After shaking for 10—15 minutes the suspension was filtered through G5 bacterium filter under sterile conditions.

In both fluid suspensions containing 100—200 million spores per ml were prepared.

To prepare *soil culture* (i) quartz sand *(arena marina,* Merck), (ii) clay, and (iii) soil sample from the own habitat of the isolate were washed and glowed. Five g of the material were placed in each of glass tubes 100 ml in length and 9 mm in diameter. The tubes were stoppered by gauze-wrapped cotton and twice sterilized at 160° C for three hours in dry air. From the sodium chloride spore suspension 0.5 ml was placed in each of the tubes containing quartz sand and clay. The same volume of the suspensions prepared in soil extracts was placed in each of the tubes with quartz sand and sterile soil obtained from the habitat. The further procedure was the same as described elsewhere [4].

As regards the conditions prevalent at the habitats two factors, *viz.* (i) the organic-matter content of the soil and (ii) the ionizing radiation of the background were studied in relation to the viability of the stored isolates.

To study the influence of the organic-matter content, viability of 18 isolates deriving from soils containing 0 to 3 per cent humus was compared to that of three isolates obtained from soils with approximately 5 per cent humus.

As to the influence of the background radiation, the viability of *Streptomyces* isolates obtained from soils containing uranium were compared to that of isolates from soils without any demonstrable background radiation. The background radiation of the former group of soils ranged from 55 to 4900 nr per hour.

"Soil cultures" were prepared from each of the isolates included in the present study. The viability of the soil cultures was studied by plating 10^{-6} diluted cultures on soy bean plates 1, 6, and 12 months after the preparation of the soil cultures. The soy bean cultures were incubated at 27° C for two days. If 100 per cent viability of the isolates had been preserved, 100—200 colonies would grow on each of the plates.

Principially, determination of the live germ count seemed to be the most reliable method to determine the quantitative changes in viability during storage.

However, the cells of the soil cultures containing clay or the soil of the habitat could not be counted in the Buerker chamber because of the incomplete sedimentation of these soils.

Consequently, the mere fact of the growth on the soy-bean agar plate irrespective of number of colonies was chosen as criterion of the viability of spores.

Results

The changes in viability of the spores of 66 isolates as determined during the first year of storage in soil cultures are shown in Table 1.

It appears from Table 1 that the cultures prepared with the original soil or with the extract prepared from the latter proved to be most viable. The

Table 1

The viability of Streptomyces *isolates in soil cultures of different preparation*

Number of isolates tested	Suspending medium	Period of storage month	Viable cultures at the end of storage in					
			quartz sand		clay		soil of original habitat	
			No.	%	No.	%	No.	%
66	0.9 per cent sodium chloride	1	49	71.2	60	90.9	—	—
	soil extract		40	60.6	—	—	59.0	89.4
	0.9 per cent sodium chloride	6	12	18.2	31.0	46.9	—	—
	soil extract		15	22.7	—	—	37.0	56.0
	0.9 per cent sodium chloride	12	4	6.0	21.0	31.8	—	—
	soil extract		6	9.09	—	—	26.0	39.3

viability of the samples stored in washed clay was not much lower. That of the samples preserved in quartz sand showed, however, a considerable decline as soon as after the first month of storage and the loss of viability was more explicit in the cultures stored for 6 or 12 months. Addition of extracts prepared from the own soil of the isolates could not suspend the inactivation of the spores.

The considerable difference that exists in the preserving capacity between clay and quartz sand appears to be attributable to the difference between these two materials as to water binding capacity.

Table 1 also shows that the isolates, in general, reserved their viability for the longest period if the spores were stored in the soil of their own habitats.

Table 2 presents comparative data on the viability of 35 isolates after storage in soil cultures. Eighteen of the isolates had derived from soils with low humus content, 17 from soils rich in humus. Viability of the cultures stored in quartz sand was with one or two exceptions essentially the same, and was not influenced by the organic-matter content of the soil of the habitats. During storage in clay the samples deriving from relatively rich soils appeared to be more viable. As a contrast, storage in the soil of the habitat was better tolerated by the *Streptomyces* strains obtained from soils poor in organic matter than by those isolated from rich soils.

In Table 3 viability of strains obtained from soils different in background radiation are compared to each other. From the data of Table 3 it appears that *Streptomyces* isolates from soil samples with irradiation background are

Table 2

Influence of the organic matter content of the habitat on the viability of Streptomyces *isolates stored in soil cultures*

Number of isolates tested	Average humus content	Period of storage month	Suspending medium	Viable cultures at the end of storage in					
				quartz sand		clay		soil of habitat	
				No.	%	No.	%	No.	%
18	1.86 (0.46—2.85)	1	Saline	12	66.6	18	100	—	—
			soil extract	12	66.6	—	—	17	94.4
		6	Saline	4	16.6	9	50.0	—	—
			soil extract	8	44.4	—	—	12	66.6
		12	Saline	1	5.55	6	27.8	—	—
			soil extract	2	11.1	—	—	8	44.4
17	3.56 (3.02—4.75)	1	Saline	13	76.3	14	83.3	—	—
			soil extract	8	47.0	—	—	13	76.3
		6	Saline	5	29.3	9	52.9	—	—
			soil extract	3	17.6	—	—	10	58.8
		12	Saline	2	11.7	8	47.0	—	—
			soil extract	1	5.89	—	—	7	41.1

in all types of soil culture more sensitive to storage irrespective of whether sodium chloride or soil extracts were used in preparation.

The enhanced sensibility to storage in soil cultures of the isolates obtained from soils with high background radiation prompted us to examine whether there is any quantitative relationship and, if there is, what kind of correlation exists, between dosage rate of background radiation and sensibility of isolate.

Table 4 provides evidence that there exists a positive correlation.

Discussion

The present studies have shown that the soil types applied in the preparation of so-called "soil cultures" considerably influence viability of Actinomyces isolates. Arena marina consisting of rather hydrophobic quartz granules seems to be least suitable for prolonged storage of the spores of Actinomyces

Table 3

Influence of the background radiation of the habitat on the viability of Streptomyces *isolates stored in soil cultures*

Number of isolates tested	Background radiation nr/hour	Period of storage month	Suspending medium	Viable cultures at the end of storage in					
				quartz sand		clay		soil of habitat	
				No.	%	No.	%	No.	%
31	4800—55	1	Saline	24	77.4	28	90.32	—	—
			soil extract	20	60.6	—	—	28	90.32
		6	Saline	3	9.67	12	38.70	—	—
			soil extract	5	16.10	—	—	14	45.16
		12	Saline	1	3.22	7	22.90	—	—
			soil extract	3	9.67	—	—	10	32.25
35	∅	1	Saline	25	71.42	32	91.42	—	—
			soil extract	20	57.12	—	—	31	88.57
		6	Saline	9	25.7	19	54.28	—	—
			soil extract	10	28.57	—	—	22	62.85
		12	Saline	3	8.57	14	40.0	—	—
			soil extract	3	8.57	—	—	16	45.71

Table 4

Influence of the intensity of background radiation on the viability of Streptomyces *isolates*

Background radiation nr/hour	Suspending medium	Per cent of viable cultures after								
		1 month in			6 months in			12 months in		
		quartz sand	clay	soil of habitat	quartz sand	clay	soil of habitat	quartz sand	clay	soil of habitat
1000—5000	Saline	66.6	73.3	—	0	26.6	—	0	6.66	—
	soil extract	73.3	—	100	6.66	—	40.0	0	—	13.3
0—1000	Saline	87.5	100	—	18.7	50.0	—	9.25	37.5	—
	soil extract	56.2	—	81.2	25.0	—	50.0	12.5	—	50.0

isolates. It appears unlikely that products extractable from the soil of habitats could considerably influence the viability of the isolate when stored in quartz sand. The viability of the cultures preserved in clay or in the soil of the habitat proved to be significantly higher as compared with cultures preserved in quartz sand. The soil of the habitat was superior even to clay when used for the preservation of spores. The cause of the different preserving capacity of these substances could not be established. Some physico-chemical characteristics, *viz.* granule size and perhaps water-retention capacity, may — among other factors — play a role in this connection.

Among the environmental factors of the soil of the habitat the humus content seems not to influence the viability of the isolate. Thus in this sense the life history of *Actinomyces* does not determine its behaviour during storage.

The background radiation of the habitat, on the other hand, considerably diminishes the tolerance of the *Streptomyces* spores to storage. Our earlier observations [6] suggest that isolates obtained from soils containing uranium are less resistant also to common damaging effects than those deriving from habitats with no significant background radiation. The direct relation between the intensity of background radiation and the decrease in viability during storage supports the above statements.

The question has arisen whether the low resistance of the *Streptomyces* isolates obtained from soils with background radiation is attributable to the possible common origin of the strains living under such conditions. However, these isolates had derived from areas remote from each other. It is hardly acceptable that such remote regions possess the same *Streptomyces* flora. Furthermore, the biochemical characteristics of the isolates obtained from radiating habitats were not identical either.

Acknowledgements

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DIE FORSCHUNG NACH ANTAGONISTISCHEN AKTINOMYCETEN IN UNGARISCHEN BÖDEN

III. Lebensfähigkeit von Streptomycesisolaten unter Reservierung in Bodenkulturen

Von den untersuchten Medien der Bodenkulturen, die zur Speicherung von Streptomycesisolaten dienen, gewannen wir bei Verwendung der eigenen Fundortböden die besten Ergebnisse. Auch der gewaschene, sterile Ton ist zur Bereitung der Bodenkulturen gut verwendbar. Der gewaschene, geglühte und sterilisierte Quarzsand ist aber zur Präparation der Bodenkulturen unverwendbar. Der Gehalt an organischen Stoffen (Humus) des Bodens der Fundorte übt keinen Einfluss auf die Lebensfähigkeit der in der Bodenkultur gespeicherten Isolate aus. Die auf dem Fundort herrschende ionisierende Hintergrundstrahlung in uranhaltigen Böden hat einen nachteiligen Einfluss auf die Lebensfähigkeit der Streptomycesstämme in Bodenkulturen.

ИССЛЕДОВАНИЕ АКТИНОМИЦЕТОВ-АНТАГОНИСТОВ В ПОЧВАХ ВЕНГРИИ

III. Выживаемость стрептомицетов во время сохранения их в почвенной культуре

При исследовании основных веществ «почвенных культур», служащих для сохранения изолированных стрептомицетов, наилучшие результаты дало использование почвы собственного места добычи. Вымытая стерильная глина тоже хорошо применима для приготовления почвенных культур. Но вымытый, прожженный и стерилизованный кварцевый песок для этой цели не пригоден. Органическое вещество (гумус), содержащееся в почве места добычи, не влияет на жизнеспособность изолированных культур во время сохранения их в почвенной культуре. Ионизирующее фоновое излучение, имеющееся на месте добычи (в почвах, содержащих уран) отрицательно влияет на жизнеспособность культуры стрептомицетов, находящихся в «почвенной культуре».

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PHYLOGENY OF HETEROSTEGININAE (FORAMINIFERA) AND PATHOLOGICAL CHANGES IN OPERCULINELLA SPECIES

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Synopsis

The material examined was supplied by the perspective deep-borings Tát No. 4, Tokod No. 350 and Tokod No. 352 (Museum of the Hungarian Geological Institute, Budapest).

Author derives the Heterosteginінae subfamily from the most ancient form of the *Operculina* genus, the *Operculina* species appearing in the Senonian stage of the Upper Cretaceous. In the present paper the change of the *Operculinella vaughani* (CUSHMAN) species belonging to the Upper Eocene Heterosteginінae subfamily of the Carpathian basin is discussed. In the phylogeny of the Heterosteginінae subfamily, the differentiation of the spiral line, the septum and the secondary septa, is illustrated.

From the spectral analysis of the *Operculinella* walls and the layers enclosing the organic remnants, relationships have been established between the incorporation of strontium into the wall and the statistical evaluation of the extent of the distortion.

The phylogenic range presented demonstrates the evolution of the Heterosteginінae subfamily.

Introduction

The *Operculina* species illustrated from South and East European regions considerably differ in their morphological forms from the American species. It was found necessary to study the *Operculina* species in European material, as the type-species of the *Operculina* genus was established by d'ORBIGNY from the Miocene layer of the Bordeaux basin. Paleontological examinations and stratigraphic results made it necessary to elucidate the geographical and chronological appearance of the *Operculina* and the derivation of the phylogeny of the Heterosteginінae subfamily descending from that genus.

These investigations led to the conclusion, that the *Operculina* species described and illustrated from European regions agree with d'ORBIGNY's (1826) description of the genus, while the "*Operculina*" species reported from American regions exhibited differences. Since the "*Operculina*" species described from American regions were traced also in Upper Eocene strata of Hungary, a comparison could be established by the material. Investigations resulted in the conclusion that the *Operculina* species determined from European and American regions belong to two different genera. The specimens published from European regions belong to the *Operculina* genus of the Nummulitinae

subfamily, whereas the *Operculina* species published from American regions to the *Operculinella* of the Heterosteginae subfamily. Phylogeny of the *Operculina* and *Heterostegina* species has been investigated from the appearance of the *Operculina*, from the Senonian stage of the Upper Cretaceous up to the Miocene, supplementing the Hungarian material by literary data (Fig. 4).

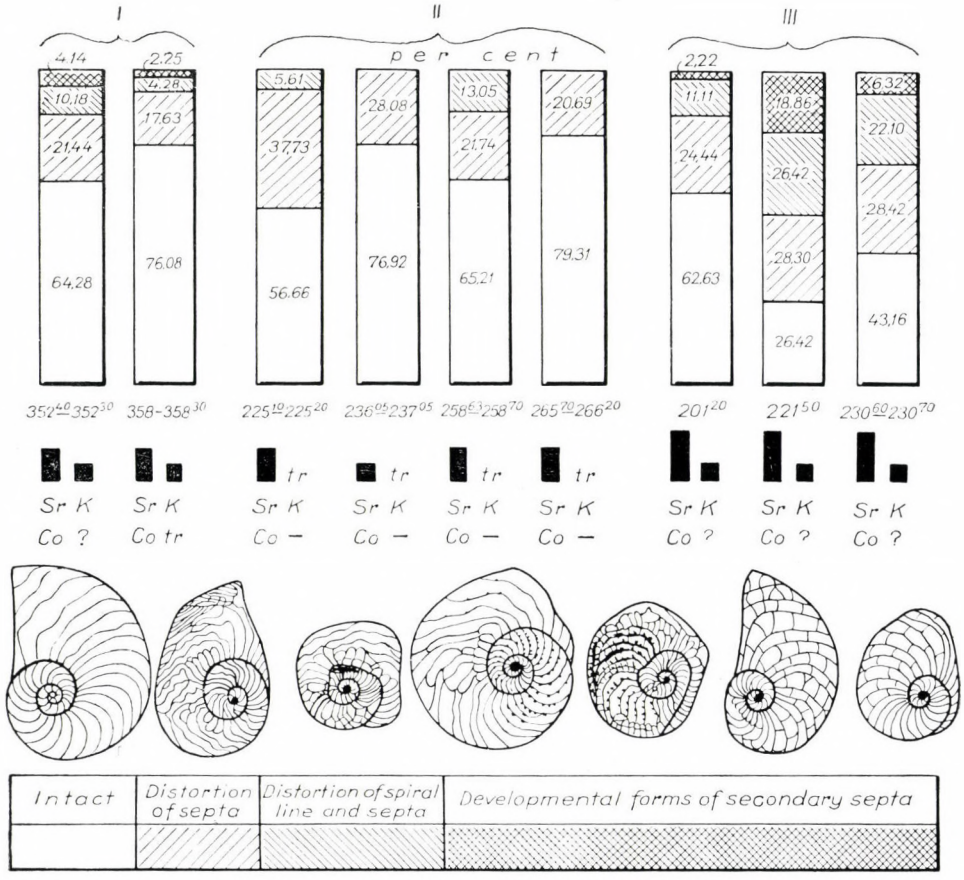

Fig. 1. Percentage distribution of *Operculinella* studied in the Upper Eocene series of the bore holes Tát No 4 (I), Tokod Nos 350 (II), and 352 (III)

The considerable changes observed in *Operculinella* species afforded the possibility for observations, by reason of which the changing features, that were traceable through phylogeny, could be outlined. The differentiation of the *Operculinella vaughani* (CUSHMAN) species occurring in the Carpathian

basin and in the flysh zone of the Carpathian Mountains, offered a chance for studying (1) development of mutation, (2) phylogeny of the Heterostegininae subfamily.

The mutation of the Operculinella species

From the shells of Foraminifera obtained from deep-borings, I made thin slices and performed the spectral analysis both of the enclosing rocks and of the Foraminifera shells elutriated of them. Investigations of the external surface and internal structure of the shell revealed the following changes in the examined *Operculinella* species: (a) on the external surface of the shell, (b) in the composition of the walls, (c) in irregular building of the shell, (d) in sexual dimorphism.

(a) On the outer surface of the shell the ornamentation is absent.

(b) From chemical analyses performed it appeared that in the substance of shells containing calcium carbonate, the calcium is partly replaced by elements having similar ionic lattice: strontium, cobalt, manganese etc. The organisms excreted extremely thin walls. In chemical analyses no shift of the calcium-magnesium ratio was observed. In these specimens a greater amount of strontium was demonstrable in the walls. Besides, a weak cobalt spectral line appeared (Fig. 1).

(c) In irregular shell construction very different forms appeared. The differentiated forms were ranged into 3 groups (Fig. 1). From the changes of forms observed the following results were obtained:

Observations	Conclusions
1. Appearance of megalospheric forms with big initial chambers.	1. Unfavourable biotope.
2. Shells of nearly identical size at the change of generations.	2. Mass decay of the microspheric forms with small initial chambers before complete development.
3. Recognition of the three degrees of distortion (Fig. 1).	3. Damage intervened in the development of septa (Fig. 2, a, b, c). The swarming isogametes were damaged and the zygots arisen from the copulation of two such isogametes produced the different forms of distortion (Fig. 2, d, e, f).

The following phenomena were established:
Disorders arising in the septa (Figs. 1 and 2).
Distortion of the spiral sheets of the walls (Figs. 1 and 2).

Development of irregular undulated and irregular secondary septa (Figs. 1 and 2).

Statistical evaluation revealed that the most distorted forms are always those with small initial chambers. Forms with big initial chambers are relatively intact in the construction of the shell disorders appear at the worst in the septa. Distortions taking place in the septa and the spiral sheets appear invariably in the microspheric B forms with small initial chambers. These forms originate from sexual reproduction. From this it was concluded, that the isogametes swarming out of the megalospheric A form with big initial chambers, were damaged and from the zygotes originating from the copulation of these isogametes, distorted specimens developed. As a result of the damage ensued in the sexual cells, the calcium carbonate secretion was considerably

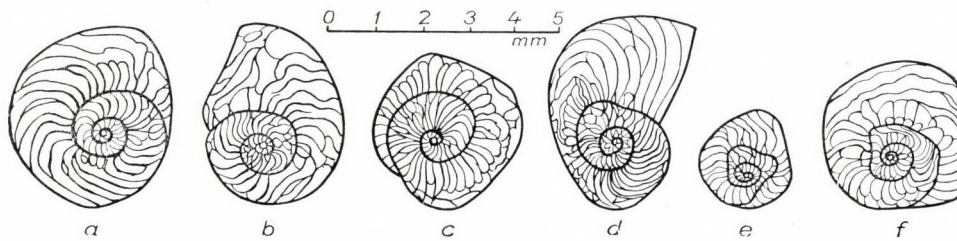

Fig. 2. Stages of the distortion in *Operculinella*

reduced in the cell, so that it was able to secrete only a very thin wall. Owing to the thin wall secretion the edge of the shell became thin and the central part (umbonal area) flattened. Such defective specimens tried to restore their balance by assuming a spherical form *i.e.* by increased sickle-like vaulting of the chambers. When even this became insufficient, and the septa were unable to connect even the distance between the spiral lines, the specimen applied a simplification by building three to four rudimentary chambers, which it failed to close off from each other, but joined them with the next chamber using an increased vault. In these distorted specimens the difference of size known in dimorphism, disappears, for the microspherical A forms ought to have attained a greater size, but exactly owing to the impairment of the calcium carbonate secretion ability necessary to the construction of shells, the full development of the specimens was inhibited. These specimens mostly perished before having attained full development and thus they remained similar in size to the megalospheric specimens with big initial chambers. On the significance of the greater proportion of strontium present in the walls of distorted specimens, thus far I failed to get an exact answer in the course of my investigations. The statistical computations showed as much, that in

the walls of the distorted specimens a greater amount of strontium was demonstrable. Here the question arises, why does the defective specimen (in reduced calcium carbonate secretion) build into its wall instead of calcium a greater amount of strontium. The investigations rather led to the supposition that the damage was brought about by the radioactive isotope of the strontium increased in connection with volcanism, and on the other hand by the effect of the Rb^{87} isotope originating from the earlier biotitic volcanism, from the decomposition of which the inactive strontium might have arisen. In the Upper Eocene seawater of the relatively well separated and enclosed basin of Esztergom the organisms with calcareous skeleton, chiefly the Foraminifera and Bryozoa, assimilated both the active and inactive strontium increased by volcanic activity. Experiments of MARTIN with a benthonic Foraminifera [8], the *Discorbis floridana* CUSHMAN revealed that in a seawater of considerable radioactive concentration the frame of the organisms exhibited high radioactive concentration. According to the same author this concentration is more frequent, chiefly in littoral benthonic organisms, which obtain their food with the detritus material of the sea.

Strontium in the walls of Foraminifera exhibits an explicit rise in the direction of Tokod—Tát (Fig. 1), tending towards the Eocene volcanic area demonstrated recently. This means that in the proximity of volcanism considerably more biotite gets into the Eocene sea. The amount of the active and inactive strontium increased on account of the volcanic activity and the decomposition of biotite transported into the seawater. It should be noted, that the Foraminifera examined by the author are also benthonic and live in the littoral seawater, their food being supplied by the organic substance of silt.

Phylogenic features of the Heterosteginae subfamily

In the course of material testing features were looked for, that occur in every genus of the Heterosteginae subfamily, but at the same time show differences during the history of the Earth. These were derived partly from the further development of existent properties, partly from the complication and reduction of the individual properties (Fig. 3):

I. Complication phenomena

1. Change of the curvature of the chambers.
2. Role of the initial chambers.
3. Development of secondary septa.
4. Tighter arrangement of chambers and secondary chambers.

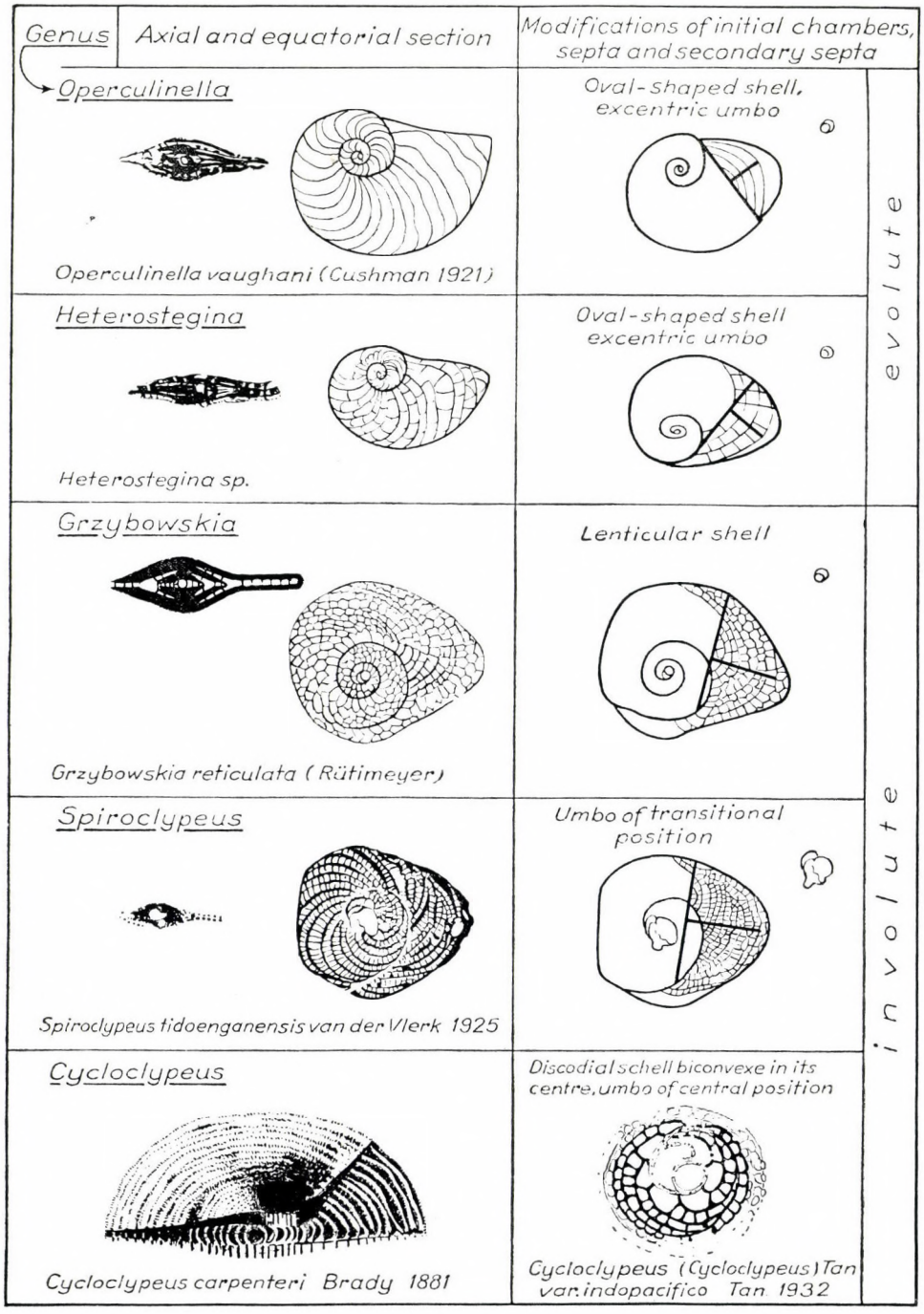

Fig. 3. **Phylogenetic features of the inner shell structure of the subfamily Heterostegininae**

II. Reduction phenomena

5. Evolute-involute character.

Operculinella is the first and simplest genus of the Heterosteginine subfamily (Fig. 4) occupying as to its morphological appearance and shell construction a place between *Operculina* and *Heterostegina*. Its phylogenetic features differ considerably from those of the *Operculina*. It exhibits a closer

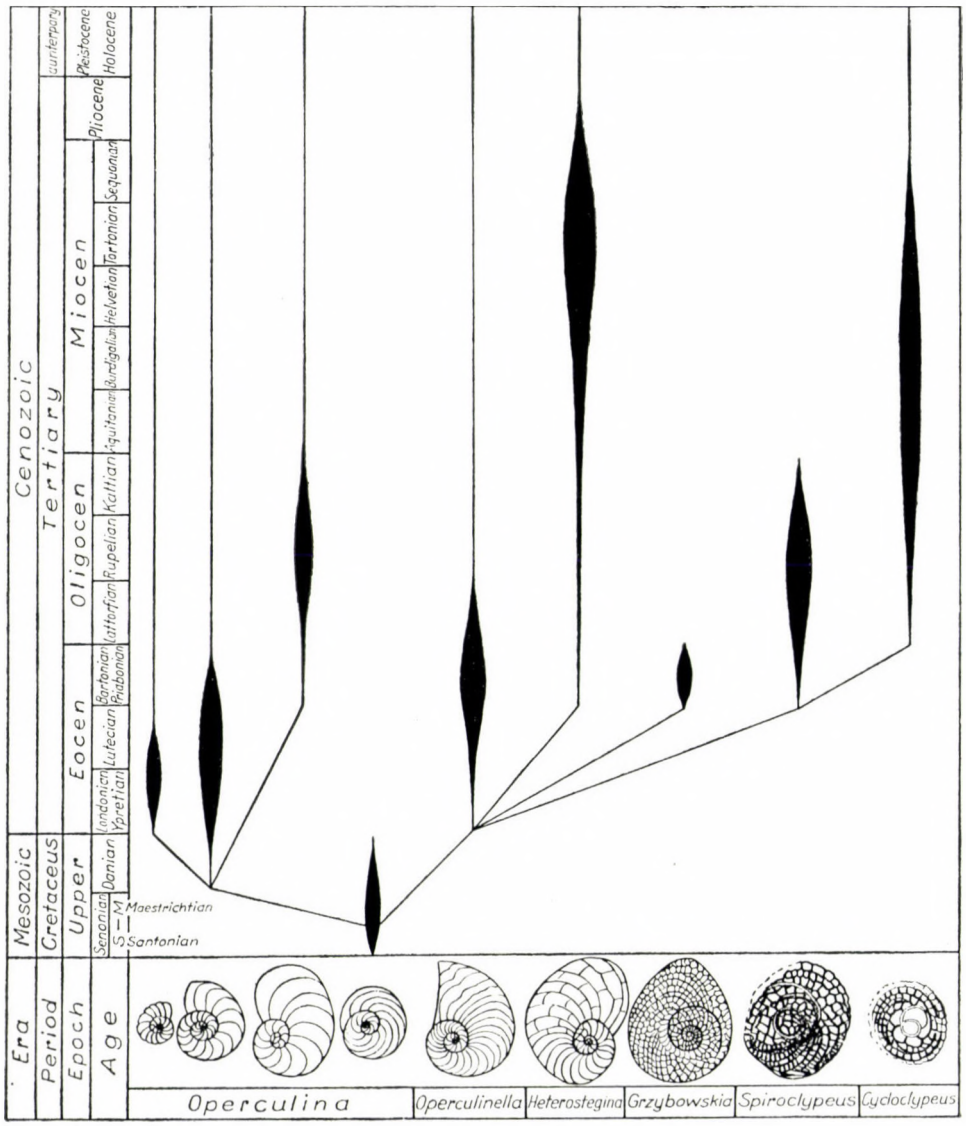

Fig. 4. Evolutionary table of the subfamily Heterosteginine

relationship to the *Heterostegina* and therefore — departing from the taxonomic system of NEMKOV [9] adopted so far — I ranged it into the Heterostegininae subfamily (Table 1).

1. I observed the considerable vaulting of the septa first in the *Operculinella ;* this feature is still more manifest in the *Heterostegina* and amounts in the *Spiroclypeus* almost to a semicircle. By the end of the developmental branch in the *Cycloclypeus*, the septa turn into a ring form, enclosing a full circle. Thus in the course of development the change of the curvature of the chambers exhibited a steadily progressive development (Fig. 3).

2. The role of the initial chambers. In the *Operculinella*, *Heterostegina* and *Grzybowskia* genera of the phylogenic line, the round initial chamber is followed by a second kidney shaped chamber. In the *Spiroclypeus* already the apparatus of initial chambers develops. Also the initial chambers exhibit a progressive development.

3. Secondary septa appear for the first time in the *Heterostegina*, by which the latter can be separated from *Operculinella*. The secondary septa of the *Heterostegina* are regular and perpendicular to the chamber septum. In *Grzybowskia* irregular secondary chambers develop with corners and partitions, which can be seen not invariably between the two septa of chambers, but often bring about accessory chamber septa consisting of several rows. In *Spiroclypeus* the secondary septum is again at right angles to the chamber septum and is arranged much more densely than in the Heterostegina. The height of the chamber septa is in every case filled up by secondary septa, situated at perfectly regular distances from each other. The chamber septa form small columns, subtending right angles (Fig. 3).

Finally it should be noted that, except for the *Grzybowskia*, in the phylogenic line a row of secondary septa was seen growing steadily denser. The Grzybowskia genus showed for this feature an instabile character, that failed to follow the trend of development, exhibiting in Upper Eocene varied forms of irregular septa.

4. Closer location of the chambers and secondary chambers appears in *Grzybowskia* and is considerably increased in *Spiroclypeus*.

Investigation of the phylogenic line of the Heterostegininae subfamily made it necessary to consider also the reductive features.

5. Evolute-involute character. The evolutional line starts with an evolute form, the *Operculinella*, showing in the *Heterostegina* a slight reduction. In the *Grzybowskia* this feature turns into an involute form and can be followed in the *Spiroclypeus* genus. Examination of the phylogenic line revealed a retrograde development of the evolute character.

I derived the Heterostegininae subfamily from the *Operculina*, where the spiral line is visible even on the outer surface of the shell. The presence of this feature can be faintly traced in the Heterostegininae subfamily on the

Table 1

Systematic divisions of the family Nummulitidae

GLAESSNER 1945 [6]	CUSHMAN 1950 [5]	POKORNY 1958 [12]	NEMKOV 1959 [9]	VITALIS-ZILAHY 1963 [15]
Nummulitidae	Camerinidae	Nummulitidae	Nummulitidae	Nummulitidae
	Archaediscinae			
	Archaediscus			
Nummulitinae	Camerininae	Nummulitinae	Nummulitinae	Nummulitinae
Operculina	*Nummulostegina*	*Assilina*	*Nummulites*	*Nummulites*
Sulcoperculina	*Camerina*	*Operculinoides*	*Operculinella*	*Assilina*
Miscellanea	*Operculinoides*	*Pellatispira*	*Assilina*	+ *Operculina*
Ranikothalia	*Paraspiroclypeus*		*Operculina*	*Neoopercolinoides*
Nummulites	*Miscellanea*		Miscellaneinae	Miscellaneinae
Assilina	*Assilina*		*Miscellanea*	*Miscellanea*
Pellatispira	*Operculinella*		*Sulcoperculina*	*Sulcoperculina*
Operculinoides	*Operculina*		*Laffiteina*	*Laffiteina*
	Sulcoperculina		Siderolinae	Siderolinae
	Heterostegina		*Siderolites*	*Siderolites*
	Spiroclypeus		*Pellatispira*	*Pellatispira*
	Heteroclypeus		*Arnaudiella*	*Arnaudiella*
	Cycloclypeus			
		Heterostegininae	Heterostegininae	Heterostegininae
		Operculina	*Heterostegina*	+ *Operculinella*
		Heterostegina	*Grzybowskia*	+ *Heterostegina*
		Spiroclypeus	*Spiroclypeus*	+ *Grzybowskia*
		Cycloclypeus	*Cycloclypeus*	+ *Spiroclypeus*
		Grzybowskia		+ *Cycloclypeus*

outer surface of the shell, but a distinct line is not visible even in the *Operculinella*.

It distinctly appears from the examination of the internal structure of the shell in the evolutional line, that in the *Operculinella*, *Heterostegina* and *Grzybowskia* genera the spiral sheet is a firm support to the shell. Besides, it controls the shape of the shell. In the older chambers of the shell of *Spiroclypeus* the spiral line still serves as a support. Later the younger chambers and the secondary chambers constitute such a strong frame as to afford a more important support to the construction. By the complete coiling of this spiral sheet, the phylogenic line arrives at its final branch, the *Cycloclypeus* (Fig. 2).

In the study of phylogeny I was concerned with dimorphism, because the comparison of the A and B forms of the same species supplied an explanation to the ranges of the Heterosteginine subfamily. In illustrating the features concerning the internal structure and shell construction of the subfamily, I employed the macrospheric A form, so that these specimens might still better illustrate the evolution and reflect at once a more advanced stage of evolution.

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PHYLOGENESE DER HETEROSTEGININAE (FORAMINIFERA) UND PATHOLOGISCHE VERÄNDERUNGEN BEI OPERCULINELLA-ARTEN

Das Untersuchungsmaterial stammt aus den perspektivischen Tiefbohrungen Tát 4, Tokod 350 und Tokod 352 (Museum der Ungarischen Geologischen Anstalt).

Verfasserin leitet die Unterfamilie Heterostegininae aus der im Senon (obere Kreide) auftretenden *Operculina*-Art, aus der ältesten Form der *Operculina*-Gattung ab. In der Arbeit wird die Veränderung der die zur Unterfamilie Heterostegininae gehörigen Art *Operculinella vaughani* (CUSHMAN) beschrieben, und veranschaulicht, wie sich die Spirallinie, die Kammerscheidewände und die sekundären Septen im Laufe der Phylogenese der Unterfamilie Heterostegininae differenziert haben.

Auf Grund der Spektralanalyse der die *Operculinella*-Schalen und organischen Reste einschliessenden Schichten wurden Zusammenhänge zwischen dem Sr-Einbau in die Schalen und den Daten der statistischen Auswertung des Deformationsgrades festgestellt. Die von der Verfasserin aufgestellte phylogenetische Reihe veranschaulicht die Entwicklung der Unterfamilie Heterostegininae.

ФИЛОГЕНЕЗ ПОДСЕМЕЙСТВА HETEROSTEGININAE (FORAMINIFERA) и ПАТОЛОГИЧЕСКИЕ ИЗМЕНЕНИЯ ВИДОВ OPERCULINELLA

Материал для исследования был взят из материала перспективных глубоких буре-ний Тат 4, Токод 350 и Токод 352 (Музей Венгерского Геологического Института).

По мнению автора подсемейство **Heterostegininae** происходит из вида *Operculina* возникшего в сенонском ярусе верхнего мела из древнейшей формы рода *Operculina* (табл. 4.). В статье излагается изменение вида *Operculinella vaughani* (CUSHMAN), относящегося к подсемейству **Heterostegininae** верхнего эоцена Карпатского бассейна. При описании филогенеза подсемейства **Heterostegininae** наглядно показывается диффе-ренциация спиральной линии, камерной перегородки и вторичных перегородок.

На основании спектрального анализа слоев, содержащих раковины и органи-ческие остатки *Operculinella*, автор обнаружил взаимосвязь между встраиванием Sr в раковины и статистической оценкой размера искажения. Установленный автором фило-генетический ряд наглядно иллюстрирует возникновение подсемейства **Heterostegininae**

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STUDIES ON ASPERGILLI

XIII. CARBON REQUIREMENTS OF SOME ASCOSPORIC MEMBERS OF THE ASPERGILLUS NIDULANS GROUP[1]

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Synopsis

The relative growth supporting values of several carbohydrates were determined quantitatively in the case of five species of the genus *Aspergillus*, *viz.*, *A. nidulans* (EIDAM) WINT., *A. rugulosus* THOM and RAPER, *A. quadrilineatus* THOM and RAPER, *A. violaceus* FENNELL and RAPER and *A. variecolor* (BERK. and BR.) THOM and RAPER. All the monosaccharides were found to be favourable sources of carbon. Sucrose and raffinose supported good growth of all the Aspergilli whereas maltose was good for *A. quadrilineatus* and *A. variecolor* and poor for the rest of the organisms. Amongst the polysaccharides, starch and dextrin supported good growth of all the moulds under study while inulin gave poor growth. Arbutin was either good, moderate or poor for the growth. Sugar alcohols supported good growth. Organic acids were useless for the growth of these Aspergilli. In general, monosaccharides, di- and trisaccharides supported either excellent or good sporulation of the majority of the present Aspergilli. Amongst polysaccharides, dextrin and starch induced good sporulation while arbutin gave poor sporulation. Organic acids were useless for the fruiting of these fungi.

Introduction

Carbon occupies a unique position amongst the essential elements required by living organism. Generally, carbohydrates are preferred by fungi as the source of carbon, but no single carbohydrate is the best source for all the fungi. However, most of the fungi can use a large variety of carbon sources for their growth and sporulation. Fungi are so specific in utilization of these substances that a carbon source may be utilized while another source of similar chemical structure may prove useless for them. STEINBERG [8] reported that *Aspergillus niger* utilized D-xylose and L-arabinose but not their enantiomorphs.

HORR [4] observed inhibitory effect of galactose and mannose on the growth of *A. niger* and *Penicillium glaucum*. TAMIYA [9] reported better growth of *A. oryzae* on arabinose than on xylose. STEINBERG [7] found D-glucose, D-fructose, D-mannose and L-sorbose to be equally effective in the nutrition of *A. niger* while D-galactose, glycerol and mannitol were poor sources of

[1] Part of a thesis approved for Ph. D. degree of Allahabad University

carbon for this organism. FERGUS [3] reported that organic acids were useless for the growth of *Penicillum digitatum*.

Literature is full of such examples which not only indicate the importance of carbon for growth and sporulation, but also establish that there is a wide difference in the types of the carbon compounds necessary for good growth of closely related organisms or even of different species of the same genus. Out of the several Aspergilli known the carbon needs of only few have been investigated. An attempt has been made here to study the effect of different carbon sources on the growth and fruiting of five moulds belonging to the *Aspergillus nidulans* group.

Materials and methods

Pure cultures of *A. nidulans* (EIDAM) WINT; *A. rugulosus* THOM and RAPER; *A. violaceus* FENNEL and RAPER; *A. variecolor* (Berk. and Br.) THOM and RAPER and *A. quadrilineatus* THOM and RAPER were selected for the present study. CZAPEK's medium minus carbon source served as the basal medium ($NaNO_3$, 3 g; KH_2PO_4, 1 g; KCl, 0.5 g; $MgSO_4 \cdot 7\ H_2O$, 0.5 g; and distilled water 1.000 ml). The different carbohydrates were supplied in amounts to yield 4.210 mg of carbon per litre. Twenty ml of the medium was taken in 150 ml Pyrex flasks. Solutions containing monosaccharides, organic acids and sugar alcohols were autoclaved at 15 lbs. pressure for 15 minutes, while fractional sterilization was carried out for di-, tri-, and polysaccharides. The flasks were inoculated by seeding ascospore suspensions of different species. They were incubated for 15 days at a temperature of $25 \pm 1°$ C. After the incubation period the content of the flasks was filtered separately and the dry weight was taken as a criterion for growth. The trial was conducted in triplicates. The results were subjected to statistical analysis in which the standard error was calculated by the formula;

$$\text{Standard Error (S. E.)} = \sqrt{\frac{\text{Mean square of the error}}{\text{Number of replicates}}}$$

and the critical difference (C. D.) was determined by the formula:

$$\text{Critical difference (C. D.)} = \text{S. E.} \times t \times \sqrt{2}$$

Experimental

The effect of various carbohydrates on the growth and sporulation of different Aspergilli is graphically represented in Fig. 1.

A perusal of Fig. 1 shows that the present Aspergilli could not grow in absence of carbon.

In general, pentoses supported good growth of all the present fungi. Only rhamnose was moderate for *A. violaceus*. Amongst hexoses, glucose and mannose were statistically poor sources of carbon. Fructose was either good or moderate for these Aspergilli. Galactose was poor for *A. variecolor* and good for the rest of the moulds. Sorbose was good for *A. nidulans*, *A. variecolor* and *A. violaceus*, moderate for *A. rugulosus* and poor for *A. quadrilineatus*.

Amongst disaccharides, sucrose supported good growth of all the present forms whereas maltose was good for *A. variecolor* and *A. quadrilineatus* and poor for the rest of the species. Lactose was good for *A. variecolor*, poor for

A. nidulans and moderate for the rest of the moulds. Raffinose gave favourable growth of all the present Aspergilli.

Starch and dextrin supported good growth of all the organisms. Arbutin was found to be good for *A. variecolor* and *A. nidulans*, moderate for *A. rugu-*

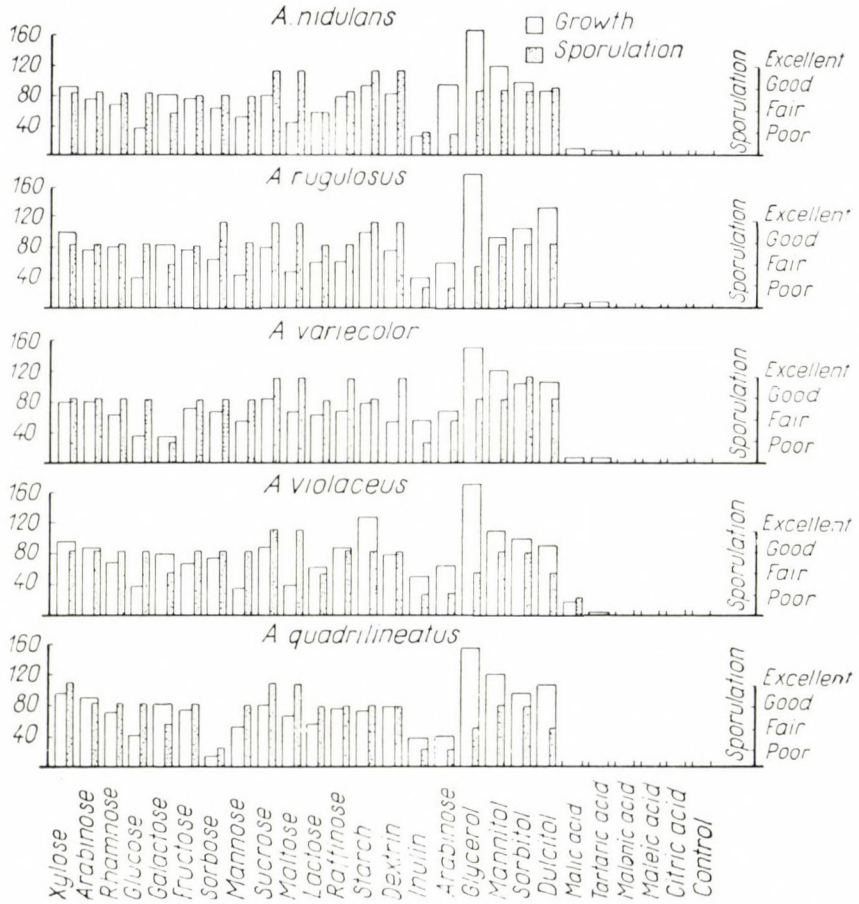

Fig. 1. Effect of different carbon sources on the growth and sporulation of five Aspergilli

losus and *A. violaceus* and poor for *A. quadrilineatus* whereas inulin supported poor growth of all the *Aspergillus* species.

All the pentoses induced good sporulation of the present fungi. Amongst the hexoses, sporulation was either excellent or good on glucose, fructose and mannose. Sorbose gave excellent fruiting of *A. rugulosus*, good of *A. nidulans* *A. variecolor* and *A. violaceus* and of *A. quadrilineatus*. Galactose, in general, induced fair sporulation in all the present fungi.

Sucrose, maltose and raffinose were excellent sources of carbon for the sporulation of these fungi. Lactose induced fair reproduction in *A. nidulans* and *A. violaceus* and good in the rest of the species.

Polysaccharides and sugar alcohols, in general, supported good sporulation while organic acids were useless for this purpose.

Discussion

Utilization of various carbon compounds depends on the ability of the fungi either to assimilate them directly or to convert the complex carbon compound into forms that can be used directly. In the latter process the enzymes associated with the organism play an important role. Those carbon compounds which can be assimilated most readily or can be oxidized with the least expenditure or energy stored in the compound appear to constitute the food of first choice for fungi.

In the present study pentoses were utilized well by these organisms. Xylose has been reported to be superior to glucose for some forms [1, 6]. Similar behaviour of xylose was also noted here. Arabinose and xylose were found to be slightly better than rhamnose. The less of growth in medium containing rhamnose was probably due to the presence of CH_3 group in its structure which is absent in the two other pentoses.

Of the disaccharides, sucrose supported good growth of these moulds whereas maltose and lactose were either good, moderate or poor for the growth. This difference in the amount of growth may be connected with the availability of the enzyme in question. Raffinose was good for all the moulds because of the general occurrence of the enzyme invertase. Similar results have been reported by TAMIYA [9] for *A. oryzae* and STEINBERG [8] for *A. niger*.

According to LILLY and BARNETT [5] the polysaccharides, in general, are soluble or only colloidally soluble. The utilization of these substances by fungi depends upon the secretion of the necessary hydrolytic enzymes. In the present investigation all the organisms utilized starch and dextrin satisfactorily. On the other hand *Penicillium digitatum* is unable to grow on starch [3]. The poor growth on inulin is probably due to the limited supply of the enzyme inulase. It has also been reported as a poor source of carbon for *A. niger* [8] and *P. digitatum* [3].

Reduction of sugars yields sugar alcohols. In the present study sugar alcohols supported excellent growth of these fungi. Good utilization of these sugar alcohols may be due to the efficient oxidation of these compounds by the present fungi. Poor utilization of mannitol and glycerol has, however, been reported by STEINBERG [8] for *A. niger*.

Organic acids are generally poor sources of carbon for fungi. In the present study also organic acids were found to be useless for the growth of

these moulds. Similar results have been reported by TAMIYA [9] for *A. oryzae*, STEINBERG [8] for *A. niger* and FERGUS [3] for *Penicillium digitatum*. There are some factors which, however, interfere with the utilization of the organic acids. Firstly, cells are often impermeable to organic acids at physiological pH levels. Secondly, the utilization of neutralized organic acids causes a rise of the culture pH which may interfere with growth. Thirdly, certain organic acids cause chelation of the inorganic ions.

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STUDIEN AN ASPERGILLI

XIII. Kohlenstoffbedarf einiger Askosporenträger der Aspergillus nidulans Gruppe

Das relative Wachstum auf Nährböden mit verschiedenen Kohlenhydraten als Kohlenstoffquellen wurde quantitativ an fünf Arten der Gattung *Aspergillus* bestimmt, d. h. an *A. nidulans* (EIDAM) WINT., *A. rugulosus* THOM und RAPER, *A. quadrilineatus* THOM und RAPER, *A. violaceus* FENNEL und RAPER und *A. variecolor* (BERK. und BR.) THOM und RAPER. Es wurde festgestellt, dass alle Monosaccharide eine günstige Kohlenstoffquelle sind. Sucrose und Raffinose gaben gutes Wachstum an allen Arten, dagegen erwies sich Maltose als gut für *A. quadrilineatus* und *A. variecolor* und schlecht für den Rest der Organismen. Unter den Polysacchariden riefen Stärke und Dextrin gutes Wachstum aller untersuchten Schimmelpilze hervor, während Inulin schlechten Wachstum ergab. Arbutin war entweder gut, mässig oder schlecht für das Wachstum. Zuckeralkohole erzeugten gutes Wachstum. Organische Säuren waren für das Wachstum dieser Aspergilli unbrauchbar. Im allgemeinen riefen Mono-, Di- und Trisaccharide entweder ausgezeichnete oder gute Sporulation bei der Mehrheit der vorliegenden Aspergilli hervor. Zwischen den Polysacchariden veranlassten Dextrin und Stärke gute Sporulation, dagegen Arbutin eine schlechte. Organische Säuren waren für die Fruchtbildung dieser Pilze unbrauchbar.

ОЧЕРКИ НАД АСПЕРГИЛЛИ

XIII. Потребление в углерод нескольких аскоспоровых форм группа — Aspergillus nidulans

Способствующее росту относительное качество различных углеводов определялось авторами количественно у пяти следующих видов рода *Aspergillus: A. nidulans* (EIDAM) WINT., *A. rugulosus* THOM и RAPER, *A. quadrilineatus* THOM и RAPER, *A. violaceus* FENNELL и RAPER и *A. variecolor* (BERK. и BR.) THOM и RAPER. Все моносахариды оказались соответствующими источниками углерода. Сахароза и рафиноза обеспечивали

хороший рост у всех изучаемых видов, мальтоза обеспечивала хороший рост у *A. quadrilineatus* и *A. variecolor*, в то время как у остальных организмов на мальтозе наблюдался слабый рост. Из полисахаридов крахмал и декстрин обеспечивали хороший рост у всех изучаемых плесневых грибков, в то время как на инулине их рост был слабым. Арбутин обеспечивал хороший, или средний, или слабый рост. Сахарные спирты обеспечивали хороший рост. На органических кислотах указанные виды *Aspergillus* не расли.

Вообще моно-, ди- и три-сахариды обеспечивали отличную или хорошую споруляцию у большинства указанных видов; из полисахаридов крахмал и декстрин индуцировали хорошую споруляцию, в то время как на арбутине обнаруживалась слабая споруляция. Органические кислоты эти грибки не способны использовать для спорообразования.

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MYELOPEROXYDASE ACTIVITY IN NORMAL RAT BONE MARROW

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Synopsis

Myeloperoxydase activity in rat bone marrow increases with the weight of the animal and runs parallel to the myeloid : erythroid ratio of the bone marrow cells. Bleeding of the animals decreases myeloperoxydase activity and also the myeloid: erythroid ratio. It seems that bone marrow myeloperoxydase activity is directly proportional to the number of myeloid cells (granulocytic series) in the bone marrow. The activity of the isolated blood leukocytes is quite similar to that of the bone marrow cells.

In 1941 AGNER [1] described an enzyme, verdoperoxydase, in human granulocytes, which is present in normal myeloid cells and in chloromas, the green tumours of the bone marrow. The enzyme, also called myeloperoxydase (MPO) is green in colour and has a peroxydative effect. It uses H_2O_2 as substrate, while the enzyme-substrate complex dehydrogenates a suitable donor molecule (aromatic amines, phenols) and H_2O develops:

Enzyme + H_2O_2 + AH_2 → enzyme — H_2O_2 + HA_2 → enzyme + A + + $2H_2O$, where AH_2 is the hydrogenated donor molecule.

MPO is a haem enzyme which contains porphyrin and iron and is inhibited by hydrogen cyanide, hydroxylamine and sodium azide. It is worth while mentioning that purified enzyme preparations also contain a small amount of copper [1].

The physiological role of the enzyme is not well understood and it is supposed to degrade the H_2O_2 developing during different metabolic dehydration processes. It seems probable that MPO is bound to cellular particulates other than mitochondria [5, 11]. SCHULTZ and co-workers [6] have compared the properties of an MPO purified according to AGNER's [1] method and those of an MPO extracted from tissues of the SHAY chloroma [8], purified by a column chromatographic method. They have also described the nature of the linkage between the peptide chain and the prosthetic group in the chloroma enzyme [7].

Histochemically MPO can be demonstrated by the use of benzidine-peroxydase and stable Nadi-reactions. According to cytological observations on smears and sections of the bone marrow and the blood, only the myeloid

elements (promyelocytes-granulocytes) and some of the monocytes give a positive myeloperoxydase reaction.

During our investigations on the SHAY chloroma of rats [3] we observed that various types of these tumours showed different MPO activities [4]. For a thorough examination of the chloroma MPO it seemed necessary to make a close study of the MPO activity of the bone marrow of normal rats. Some preliminary results have been described elsewhere [4].

In the present study we have determined the MPO activity in the bone marrow of normal rats of different weight and observed that it increases with the weight (age) of the animals and runs parallel to the myeloid : erythroid ratio of the bone marrow cells. Comparison of the MPO activities of the mature blood granulocytes of animals of different weight showed that the values for the older animals equalled those for younger ones.

Methods

Preparation of the homogenates. Albino rats of our random bred strain were kept on a diet consisting of maize, oats and powdered milk, casein, meat meal, ol. Helianthi, ol. Lini, baker's yeast, carrots, NaCl, $CaCO_3$, sprouting wheat grains and the preparation "Erra" (Állami Vaccinatermelő Intézet, Budapest, Hungary) containing oxytetracycline, vitamin B_{12} and amino acids in rice bran.

The animals were killed by cervical dissection under light ether anaesthesia. Both femurs were exposed and their shafts opened with a sharp knife. The bone marrows were collected, put in glass stoppered siliconized small flasks containing 2 ml of 0.05 *M* tris(hydroxy) methylaminomethane (Tris) buffer at pH 7.0 and weighed. Then Tris-buffer was added so that a bone marrow tissue concentration of 4—10 mg/ml was achieved. The marrow suspensions were homogenized in a motor-driven all-glass Potter-type homogenizer for two minutes. After homogenization the homogenates were diluted to 1 mg/ml tissue concentration. MPO activity was determined by the method previously described [4] and expressed in units/100 mg wet tissue.

Myeloid: erythroid ratio. Bone marrow smears were prepared, fixed in methanol, stained with Giemsa solution, and 200 cells counted. The averages of the myeloid : erythroid ratio in the bone marrow of rats whose bone marrow MPO activity was determined is given in Table 1. These values are somewhat higher than those of SHEN and HOSHINA [9] and BURKE and HARRIS [2].

Bleeding experiments. Animals weighing 190—200 g were bled by cardiac punctures. The blood loss was 0.58—0.85 ml/100 g body weight/day for 7—10 days. In the bone marrow smears of such animals the myeloid : erythroid ratio was reduced to 0.27—0.50 showing an increase in number of the erythroblasts.

Isolation of the blood leukocytes. 4—6 ml blood was withdrawn by cardiac puncture from rats weighing 100—170 and 250—300 g with "Liquoid" (Roche). The blood samples were poured into test tubes containing 4—6 ml of 6% dextran solution in saline ("Intradex", Crookes Lab.) and allowed to stand until settled. After the majority of the erythrocytes had settled the upper leukocyte-rich suspension was sucked off, centrifuged at 100 g for 10 minutes, resuspended in Tris-buffer and homogenized. It should be mentioned that erythrocytes do not exhibit any appreciable MPO activity [10]. MPO activity was determined in the same way as in the case of bone marrows and expressed in units per 10^7 neutrophil and eosinophil granulocytes. The supernatant of the 100 g centrifugate did not show any measurable activity. The number of the isolated blood leukocytes was determined in quadruplicate samples and each sample counted twice. The standard error of the counts was $\pm 10\%$ of the total cell count. The smears were prepared from leukocyte suspensions, fixed with methanol and stained with Giemsa solution. From each suspension two smears were prepared and 200 cells counted. The percentage distributions of the neutrophilic and eosinophilic granulocytes and ymphocytes were determined.

Results

The MPO activities in the bone marrow of 26 rats weighing 20—80 g, of 14 weighing 81—180 g and of 17 weighing 181—300 g were determined. In Table 1 the number, sex and weight of the animals, the myeloid: erythroid ratios and the means, standard errors and ranges of the MPO activities in the bone marrows are given as the results of a statistical analysis. As may be seen, the MPO activity of the bone marrow increases with weight and is parallel to the myeloid: erythroid ratio. The differences in MPO activity in relation to the weight of the animals seem to be significant. Sex-related differences could not be demonstrated. In a few animals weighing 180—300 g the MPO activities in both femoral bone marrows were determined individually, but no significant differences were found.

Table 2 shows the MPO activities of the bone marrows of 7 rats with blood losses of 0.58—0.85 ml/100 g body weight/day for 7—10 days. The average MPO activity of the bone marrows is about one third of that of the healthy animals; the same is true for the myeloid : erythroid ratios.

In Table 3 the means, the standard deviations, and the ranges of the MPO activities of isolated blood leukocytes for two groups of rats are given. Each group consisted of 6 animals, weighing 100—170 and 250—300 g, respectively. There is no significant difference in activity of the isolated blood leukocytes between the two groups.

Table 1

Myeloperoxydase activity of the bone marrows of rats of different ages

No.	Number of animals	Sex		Weight of animal (g)	MPO activity in units/100 mg wet bone marrow tissue, means, ±SD (ranges)	Myeloid: erythroid ratio	Significance of the differences
		Male	Female				
(1)	26	11	13	20— 80	86 ± 42 (26—170)	0.49	(1) vs (2) $p < 0.001$
(2)	14	6	8	81—180	145 ± 58 (62—287)	1.23	(2) vs (3) $0.02 > p > 0.01$
(3)	17	10	7	181—300	230 ± 104 (88—461)	1.60	(1) vs (3) $p < 0.001$

Discussion

In our experiments we found the MPO activities of the rat bone marrows to show definite differences which seemed to be related to the weight (age) of the animals. Searching for the causes of the low MPO activity in young, and for those of the higher activity in older rats we found that the bone marrow

Table 2

Myeloperoxydase activity of the bone marrows of rats bled for 7—10 days

No.	Weight and sex (g)	Blood loss (ml/day/100 g body weight)	Duration of bleeding	MPO activity of bone marrow (units/100 mg wet tissue)	Myeloid: erythroid ratio
1	280 female	0.58	7	83	0.27
2	280 male	0.57	7	50	0.28
3	260 female	0.79	7	108	0.45
4	210 female	0.85	7	93	0.46
5	240 female	0.85	7	97	0.46
6	210 male	0.90	10	80	0.38
7	190 male	0.78	10	41	0.50

Table 3

Myeloperoxydase activity of blood neutrophils and eosinophils in two groups of rats with an average body weight of 265 and 135 g respectively

Number of animals	Weight (g), mean, ±SD (ranges)	Number of isolated blood leukocytes (10^6 cells/ml) Tris-buffer, means, ±SD (ranges)	Percentage of neutrophils and eosinophils, means, ±SD (ranges)		MPO activity (units/10) neutrophils and eosionophils, means, ±SD (ranges)
			Neutrophils	Eosinophils	
6	265±25 (250—300)	4.2±1.2 (2.5—6.0)	10.7 ±1.2 (10—13)	1 ±0.6 (0—2)	20.8 ±4.6 (13.4—27.2)
6	135±25 (100—170)	3.8±0.8 (2.6—4.7)	11.3 ±3.4 (7—16)	2.7 ±2 (0—5)	19.0 ±4.0 (13.7—25.4)

The difference in MPO activity between the two groups is not signif icant ($0.5 > p > 0.4$)

MPO activities ran parallel to the myeloid : erythroid ratio, *i.e.*, the number of myeloid cells might be responsible for the differences in activity. The same conclusion may be drawn when our cytological findings and enzyme activity values are compared with the data of BURKE and HARRIS [2], who determined the number of bone marrow cells and their percentage distribution in rats of different ages. These authors [2] examined rats of 1 to 50 weeks of age and found, as we did, that the number of myeloid cells of the bone marrow increased with the weight of the animals.

That the myeloid cells of the bone marrow are responsible for MPO activity and that their rate of activity is determined by their number is further demonstrated by our bleeding experiments. Bone marrow erythroblastosis (decrease of the myeloid : erythroid ratio to 0.27—0.50) produced by bleeding, was accompanied by a decrease in MPO activity by about a third of the control values.

It seemed to be of great interest to determine the MPO activity of some types of the granulocytic series. For that purpose the activity of mature blood granulocytes (neutrophils and eosinophils) isolated from the blood with dextran was examined. No weight (age)-related difference was found.

A comparison between the activity of the bone marrow and that of isolated blood granulocytes was made on the basis of the data of BURKE and HARRIS [2]. They examined the number of cells per unit of weight in the wet bone marrow of the rat and found that there were about two million cells in 1 mg wet bone marrow tissue. They also observed that about 50% of all cells were myeloid elements (granulocytic series). Since 10 mg wet bone marrow tissue (*i.e.* 10^7 myeloid cells) displays an MPO activity of about 23 units, and 10^7 blood granulocytes an avarage activity of 20 units, it can be said, that the activity of the bone marrow myeloid cells (mainly immature cells) is similar to that of the mature blood granulocytes.

It should be mentioned that, according to our uncompleted experiments, dextran inhibits to some extent the MPO activity of the bone marrow. Mature granulocytes isolated from the peritoneal cavity, however, are not inhibited by dextran, when treated in the same way as blood granulocytes. Thus, there might be a difference in sensitivity to dextran between mature and immature cells.

From our data it is impossible to estimate the MPO activity of myeloid cell types other than mature granulocytes. The determination of MPO activity of other cell types of the granulocytic series could be achieved by separating the bone marrow myeloid cells or rather by provoking a reactive bone marrow change with the predominance of some types of the granulocytic series. That will be the purpose of further investigations.

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MYELOPEROXYDASEAKTIVITÄT IM KNOCHENMARK DER NORMALEN RATTE

Die Myeloperoxydaseaktivität im Knochenmark der Ratte steigt mit dem Gewicht des Tieres an und verläuft parallel zum Verhältnis der myeloiden und erythroiden Zellen des Knochenmarkes. Blutung der Tiere vermindert die Myeloperoxydaseaktivität sowie das myeloid-erythroide Verhältnis. Es scheint, dass die Myeloperoxydaseaktivität des Knochenmarkes sich zur Zahl der myeloiden Zellen (Granulozytenreihe) im Knochenmark direkt proportional verhält. Die Aktivität der isolierten Leukocyten des Blutes ist derjenigen der Knochenmarkzellen ähnlich.

АКТИВНОСТЬ МИЭЛОПЕРОКСИДАЗЫ В КОСТНОМ МОЗГУ У НОРМАЛЬНЫХ КРЫС

Активность миэлопероксидазы в костном мозгу крыс повышается с увеличением веса животных и изменяется параллельно миэлоидно-эритроидным отношениям костномозговых клеток. Кровопускание животных приводит к снижению активности миэлопероксидазы, а также к уменьшению миэлоидно-эритроидного отношения. Оказывается, что активность миэло, пероксидазы прямопропорциональна количеству миэлоидных клеток (ряда гранулоцитов) в костном мозгу. Активность лейкоцитов, изолированных из крови, такая же, как активность костномозговых клеток.

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AN OUTPOST SITE OF CORYNEPHORUS CANESCENS IN THE REGION BETWEEN THE DANUBE AND THE TISZA AND ITS CAUSAL EXPLANATION

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Synopsis

The paper deals with the causal explanation of the occurrence of *Corynephorus canescens* in the region of Nagykőrös. Evaluation of the site factors of the forest complex "Nagyerdő" proved that soil acidity represents the limiting factor in the distribution of this plant. To establish its acidity demands, a method indicating the regulation power of the root system was applied. It appeared that in unbuffered medium the acidity amplitude of the regulation power ranges from 3.4 to 9.2 pH with an optimum around 5.8 pH. Simultaneously the soils under the following closely adjacent plant associations were examined:

Festucetum vaginatae danubiale corynephoretosum canescentis,
Festucetum vaginatae salicetosum rosmarinifoliae,
Festucetum vaginatae danubiale.

The buffer capacity and reducing effect of these soils were established. Thus the supposition could be confirmed that in the examined region *Corynephorus canescens* grows only in sites where buffer capacity and reducing effect of the soil are minimal. The plant does not occupy soils of higher buffer capacity which cannot be regulated by its root system.

Introduction and problems

Studying the causal phytogeography of *Corynephorus canescens*, the author had to cope with the problem of substratum acidity constituting — beside climatic conditions — an important limiting factor in the distribution of this psammophilous plant. *Corynephorus canescens* may be commonly found on acidic sandy soils of Central Europe but avoids the large alkaline sand complexes of the region between the Danube and Tisza rivers in Hungary, although, as to its climate and physical properties of the substratum, this region may be looked upon as a potential area of *Corynephorus canescens* in compliance with BOYSEN-JENSEN [1]. This is corroborated by the fact that *Corynephorus canescens* does appear in the region, but only in some restricted areas, *e.g.* near Nagykőrös [5]. An attempt at explaining the fact that its presence is confined to sporadic and small patches of land was made as early as 1940 by HARGITAI [3], who pointed to the neutral or moderately acid soil reaction differentiating these localities from the basic calcareous sands in other parts of the region. By the courtesy of the Hungarian Academy of Sciences, in the autumn of 1961

the author could obtain a deeper insight into this highly interesting problem of ecology and phytogeography.

A thorough investigation of the acidity demands of *Corynephorus canescens* in Czechoslovakia showed that the plant can regulate the unbuffered substratum by its root system within a relatively wide range, approximately between the pH-values of 3.5 to 10.0 towards the optimum values ranging from 5.5 to 5.9 pH. Its natural occurrence in Czechoslovakia was also found to comply with the latter figures [7]. Thus it is potentially possible for this plant to grow on sandy soils of 7 to 8 pH, if they possess a minimum buffer capacity. HARGITAI found the pH-values for soils under Festuceto-Corynephoretum in Nagykőrös as ranging from 6.92 to 7.89. Whether these were indeed sandy soils of minimum buffer capacity or a special ecotype of *Corynephorus canescens* had developed with somewhat different acidity demands — this was a question which could be solved by experiments only. The author thus examined the ability of plants to control the pH-value of the soil by their roots and also directed her research towards the buffering and "reducing" effect of the soils near Nagykőrös and for comparison on the Szentendre Island of the Danube, following the method of ÚLEHLA and MARTINEC [8, 9, 10]. (The "reducing" effect of the soils manifests itself in the changes of buffering capacity in the course of time.) All these factors participate in the establishment of a dynamic equilibrium between plant and soil; their analysis should contribute to the causal explanation of the occurrence of the plant under consideration.

Material and methods

With the assistance of dr. VERA KÁRPÁTI and Dr. MARGARET KOVÁCS two patches of ground covered predominantly with *Corynephorus canescens* were chosen in the complex of the Nagyerdő Forest near Nagykőrös and in its close vicinity other patches with predominating *Festuca vaginata* and lacking entirely in *Corynephorus canescens* were marked off. According to the sociological surveys made by V. KÁRPÁTI and M. KOVÁCS the vegetation of these areas could be defined in accordance with the classification of HARGITAI [3] as well developed plant associations. For comparison experimental material of sandy soils of the Szentendre Island was also examined; it was collected between Pócsmegyer and Szigetmonostor from two localities similarly covered with typically developed associations. The characteristic data of all localities are presented in Table 1.

In the above-mentioned plant associations soil samples from the rhizosphere of the dominant plants and in the localities 1 and 2 also complete individuals of *Corynephorus canescens* were collected; the latter were brought into the laboratory very carefully and examined experimentally on the next day.

The regulative power of the plants was studied according to a previously described method [6, 7]. The whole root system was divided into six equal parts and each of these put for two hours into an unbuffered solution of a fixed pH-value obtained by adding hydrochloric acid or potassium hydroxide to distilled water. The acidity of the solution was measured colorimetrically by means of the universal indicator Čůta-Kámen (a Czechoslovak make) before and after the experiment. This procedure was repeated six times.

Establishment of the *buffer capacity of the soil.* The airdried soil samples were treated in the laboratory for several weeks. Generally the method described by BRAUN-BLANQUET [2] was applied. The fine soil samples weighing 10 g each were doused with the following solutions

16—8—4—2—1—0 ml	0.01 n hydrochloric acid	(F = 0.984)
16—8—4—2—1—0 ml	0.01 n sodium hydroxide	(F = 1.062)

Table 1

Data of localities studied

No.	Locality	Plant association	Dominant plants	Remarks
1	Nagyerdő	Festucetum vaginatae danubiale corynephoretosum canescentis	*Corynephorus canescens*	
2	Nagyerdő	Festucetum vaginatae danubiale corynephoretosum canescentis	*Corynephorus canescens*	Mostly one-year old seedlings
3	Nagyerdő	Festucetum vaginatae salicetosum rosmarinifoliae	*Festuca vaginata* *Stipa joannis*	On the slope of a dune
4	Nagyerdő	Festucetum vaginatae danubiale	*Festuca vaginata*	
5	Szentendre-Sziget	Festucetum vaginatae danubiale	*Festuca vaginata* *Phleum phleoides*	
6	Szentendre-Sziget	Festucetum vaginatae danubiale	*Festuca vaginata*	

and filled up to 100 ml with distilled water. Poured into glass-stoppered vessels and occasionally shaken, these soil suspensions were left standing for 24 hours and subsequently their pH-value was measured with a glass electrode. As standard for comparison a similarly prepared suspension of sea-sand pro analysi was employed.

Determination of the reducing effect of the soil means in principle the introduction of the time factor into the establishment of buffering capacity. Here again the method described under "buffer capacity of the soil" was applied but the process of restoring the acidity equilibrium was registered 10 minutes, 1, 2, 4, 8, and 24 hours after introducing the soil into solutions. Further details of this procedure may be found in the paper of ÚLEHLA and MARTINEC [8, 9, 10].

Results

Data pertaining to the regulative power of the plant are plotted in Fig. 1. It is shown there that the regulation point indicating the optimum pH-value to which the unbuffered solutions are adapted by the plant lies at 5.8 pH. The range of the regulation zone represented by the pH-values of 3.4 to 9.2 on the abscissa corresponds to the amplitude of acidity within which the unbuffered medium can be regulated by the plant toward its optimum.

The buffering of soil suspensions prepared from the material of the localities 1 to 6 and examined after 24 hours is characterized by the curves in Fig. 2. These graphs demonstrate that both soils taken from the rhizosphere of *Corynephorus canescens* in Festucetum vaginatae danubiale corynephoretosum canescentis (1, 2) have a relatively low buffer capacity, being nearly equal with that of the suspension of seasand pro analysi in acid medium. The soil under Festucetum vaginatae salicetosum rosmarinifoliae (3) has a higher buffer capacity although the values of the actual acidity — measured in aque-

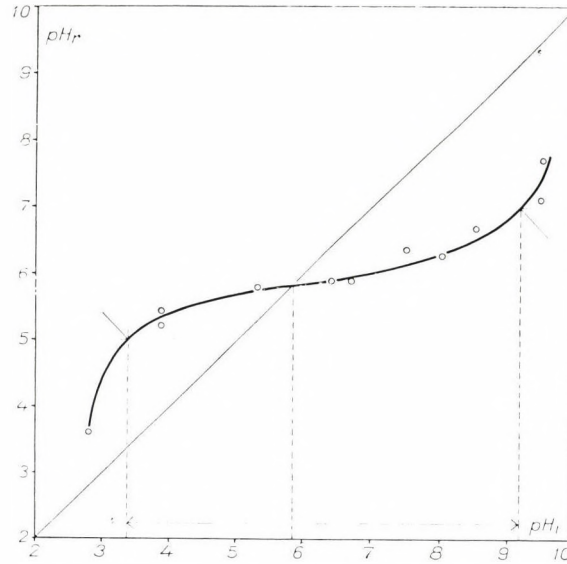

Fig. 1. Regulation power of the root system of *Corynephorus canescens* from the "Nagyerdő" forest. Mean curve of six measurements. Abscissa: initial pH-value. Ordinate: regulated pH-value

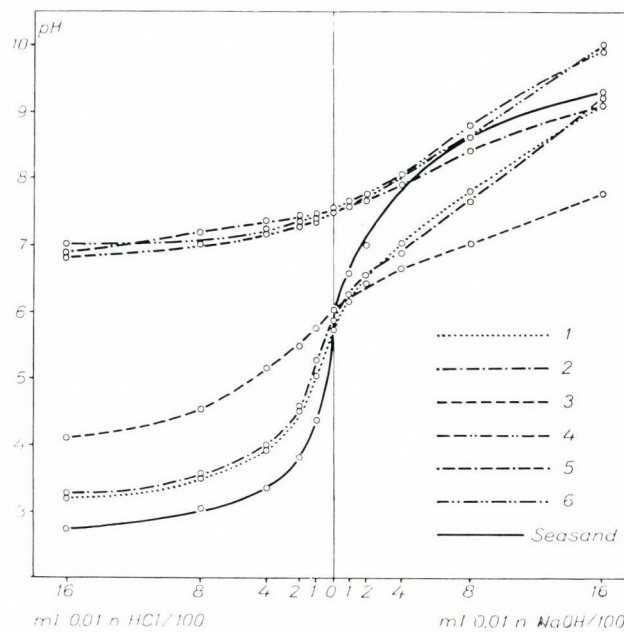

Fig. 2. Buffer capacity of soils taken from the rhizosphere of psammophytes compared with the buffer capacity of a suspension of seasand pro analysi. Numbers indicate the sites. Abscissa: 0.01 n hydrochloric acid or sodium hydroxide in ml per 100 ml of the solution. Ordinate: pH of the suspension after 24 hours

ous suspension — do not considerably differ from those of the two soils mentioned above. The other three nearly identical curves belong to 3 samples taken from the association Festucetum vaginatae danubiale although in three different localities (4, 5, 6). Their buffer capacity is high, especially in acid medium.

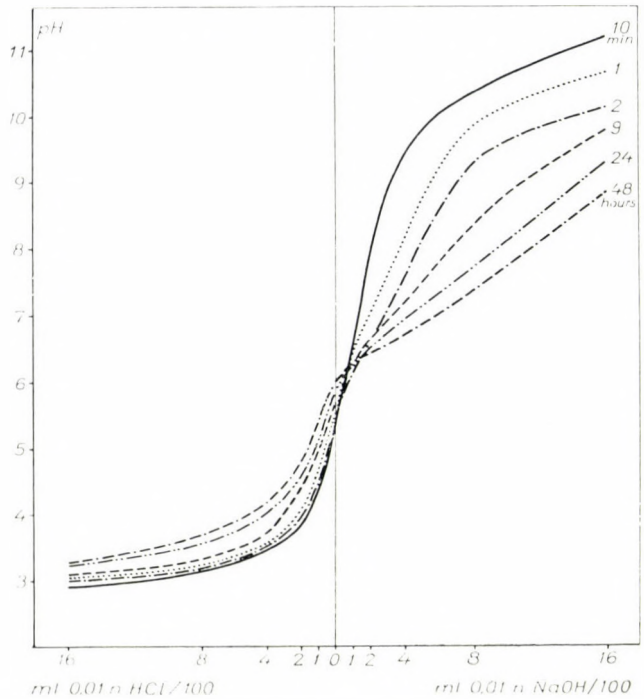

Fig. 3. Reducing effect of the soil from the rhizosphere of psammophyte vegetation in "Nagyerdő". Soil sample from the association Festucetum vaginatae danubiale corynephoretosum canescentis. Abscissa: 0.01 n hydrochloric acid or sodium hydroxide in ml per 100 ml of the solution. Ordinate: pH of the suspensions; the curves correspond to the time period of the examined process

The reducing capacity of soil suspension is illustrated graphically in igs. 3, 4, and 5. For this purpose only soils from the forest "Nagyerdő" were chosen, their vegetation corresponds to the three analysed plant communities (1, 3, 4). The reducing capacity of the other soils is analogous, which may be after all found from the curves of the buffer capacity. Since, however, the time factor plays a decisive role in the buffering process of the suspension, this process should be represented in three dimensions. Still, the required clearness of demonstration may be ensured by extrapolation carried out in one optional point only. Thus from the curves represented in Figs. 3 to 5 the extrapolation was performed for the values 8 ml 0.01 n hydrochloric acid per 100 ml, con-

sidering time as the variable quantity. Fig. 6 shows the graphical illustration of the result, demonstrating the complicated reducing process in a soil system with its acidity equilibrium upset.

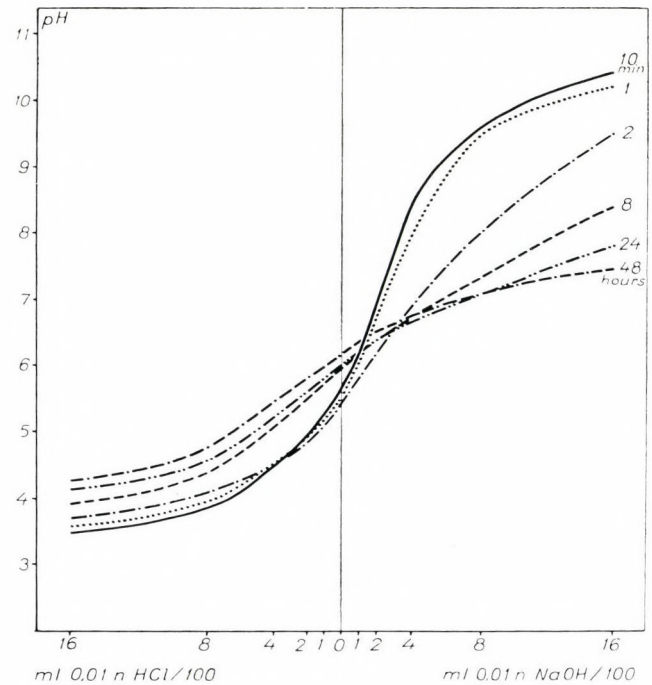

Fig. 4. Reducing effect of the soil from the rhizosphere of psammophyte vegetation in "Nagyerdő". Soil sample from the association Festucetum vaginatae salicetosum rosmarinifoliae. Abscissa: 0.01 n hydrochloric acid or sodium hydroxide in ml per 100 ml of the solution. Ordinate: pH of the suspensions; the curves correspond to the time period of the examined process

Discussion

If we try to establish causal relations between the distribution of some plants and soil acidity, we cannot content ourselves, despite the highest precision in measurement, with a pH-value only, even though it may be the result of a measurement carried out in an apparently dynamical manner during the vegetation period. In the localities under study a coincidence between the presence of *Corynephorus canescens* and the initial acidity could be hardly found; we measured the following pH-values in the various localities (in ten minutes intervals):

No. of site:	1	2	3	4	5	6
pH:	5.50	5.62	5.65	6.23	8.01	6.78

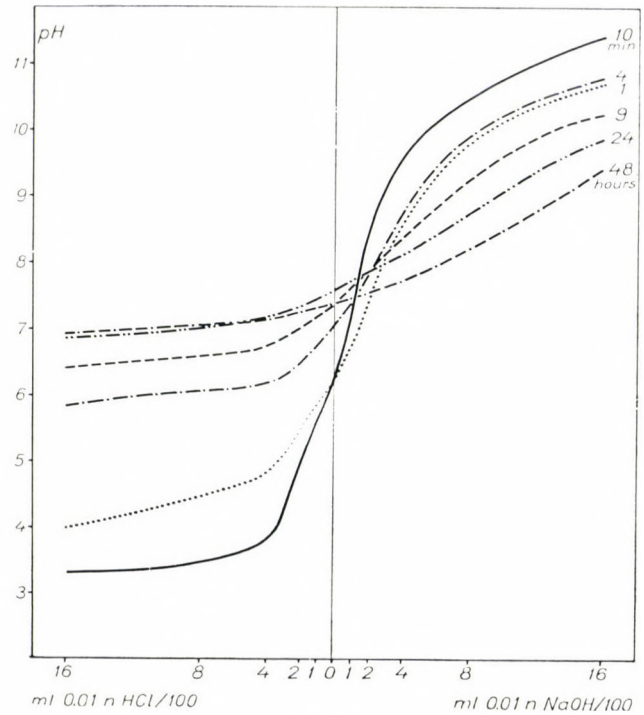

Fig. 5. Reducing effect of the soil from the rhizosphere of psammophyte vegetation in "Nagyerdő". Soil sample from the association Festucetum vaginatae danubiale. Abscissa: 0.01 n hydrochloric acid or sodium hydroxide in ml per 100 ml of the solution. Ordinate: pH of the suspensions; the curves correspond to the time period of the examined process

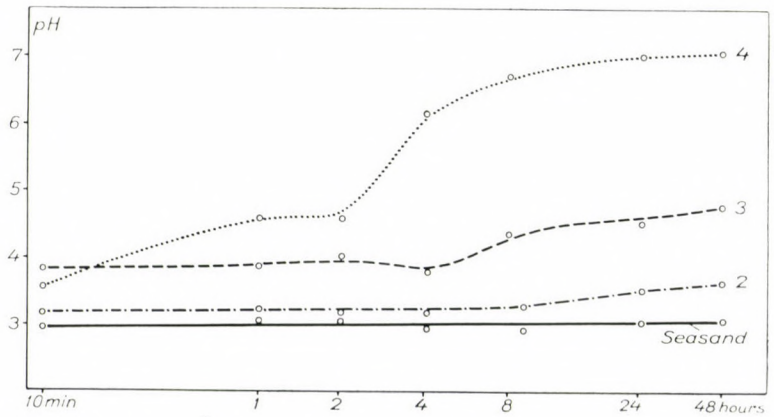

Fig. 6. Reducing effect of soils from the psammophyte vegetation of the "Nagyerdő" as related to the time factor. Abscissa: time (logarithmically) in hours. Ordinate: pH of the suspension. Extrapolation for suspensions of 0.8 ml 0.01 n hydrochloric acid

The occurrence of *Corynephorus canescens* was, however, established only in the localities 1 and 2, whereas the plant species constituting the vegetation of the other localities belonged to other associations. It is therefore necessary to assess soil acidity more comprehensively, even if the components of this complex need not be analysed. One of the methods supplying information on the pattern of soil reaction is the common method establishing the soil buffering. Fig. 2 reveals at a glance to which ecological groups the examined soils belong. Those in localities 1 and 2 are unbuffered soils, No. 3 shows moderate buffering, whereas the material from localities 4, 5, and 6 represent highly buffered soils. It is not accidental that *Corynephorus canescens* occupies only the areas of low buffer capacity in this region although all the other conditions in the localities are almost identical in the entire forest complex. Whether the low buffer capacity is due to the relief, the soil profile or the previous vegetation, cannot be answered in the present paper. The determination of buffer capacity discloses only the final stage reached by the soil suspension after 24 hours, thus being a quantitative measure, revealing nothing about the qualitatively different course of the process as to the time factor. The study of the reducing effect of the soil is of much importance exactly at this point. It shows that the soil suspension is not only a buffer system analogous to aqueous solutions, which change their pH-values after the admixture of acids or bases and sooner or later reach their equilibrium according to the composition of the solution. The soil suspension represents a complicated heterogeneous system in which the regulation principle makes itself felt. This means that the system, if transformed by external interference, returns in a new process into its original state [8, 9, 10]. The validity of this principle, which is an important factor in the ecology of the examined species, is demonstrated in Fig. 6. The soil from Festucetum vaginatae danubiale of the Nagyerdő, when acidified, returns to its original pH-value in the course of a few hours. In comparison with this soil, that of Festucetum vaginatae salicetosum rosmarinifoliae shows a lower reducing power, although its buffer capacity is of medium value. From these facts it may be concluded that after some climatically favourable years promoting its vitality *Corynephorus canescens* would also occupy this part of the locality. The soil under Festucetum vaginatae danubiale corynephoretosum canescentis is characterized not only by insignificant reducing power but also by very low buffer capacity. The occurrence of *Corynephorus canescens* is thus quite regular here, because if the other ecological conditions are favourable, there is no obstacle hindering the plant in its adaptation to optimum acidity. It was sufficiently proved by the experimental investigation of the regulative power of roots that in the case of plants on the outpost site in the region between the Danube and the Tisza no adapted ecotype is involved. The run of the regulation curves is identical with that of the graphs established

for the same plants in Czechoslovakia, both in the region of the Morava Valley and in Elbe Valley [7].

The results of researchwork reported here have proved again that certain phytogeographical questions may be causally solved on the basis of a thorough ecological analysis by envisaging the whole problem dynamically and considering both factors which prevail in the occupation of localities, *i.e.* both the habitat and the plant.

My thanks are due to the workers of the Botanical Institute of the Hungarian Academy of Sciences and to Professor B. Zólyomi for enabling me to perform all the investigation. I am especially indebted to Mrs. Vera Kárpáti for her valuable cooperation.

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DER VORPOSTENSTANDORT VON CORYNEPHORUS CANESCENS IM DONAU—THEISS-ZWISCHENSTROMGEBIET UND SEINE KAUSALE ERKLÄRUNG

Die vorgelegte Arbeit befasst sich mit einer kausalen Erklärung des Vorkommens von *Corynephorus canescens* im Gebiete von Nagykőrös. Bei Beurteilung der Standortsfaktoren des Waldkomplexes Nagyerdő zeigte es sich, dass die Bodenaziditätsverhältnisse hier den Grenzfaktor der Verbreitung dieser Pflanze bilden. Zur Feststellung der Aziditätsan-

sprüche der Pflanze wurde eine die Regulationsfähigkeit des Wurzelsystemes anzeigende Methodik verwendet. Es zeigte sich, dass sich die Aziditätsamplitude des Regulationsvermögens im ungepufferten Medium von 3,4 bis 9,2 bewegt, mit einem Optimum um pH 5,8. Zugleich wurden auch Böden unter folgenden, eng angrenzenden Assoziationen geprüft:

Festucetum vaginatae danubiale corynephoretosum canescentis
Festucetum vaginatae salicetosum rosmarinifoliae
Festucetum vaginatae danubiale.

Es wurde ihr Pufferungs- und Dämpfungsvermögen bestimmt. Auf diese Weise wurde die Voraussetzung bestätigt, dass in diesem Gebiet die Pflanze nur dort vorkommt, wo die Bodenpufferung und Bodendämpfung minimal ist. Böden mit grösserer Pufferung, welche die Pflanze durch ihr Wurzelsystem nicht zu regulieren vermag, werden von ihr nicht mehr besiedelt.

МЕСТОНАХОЖДЕНИЕ CORYNEPHORUS CANESCENS В РАЙОНЕ МЕЖДУ ТИССОЙ И ДУНАЕМ И ЕГО КАУЗАЛЬНОЕ ОБЪЯСНЕНИЕ

Данная работа занимается каузальным объяснением нахождения *Corynephorus canescens* в районе г. Надькёрёш. При оценке факторов произрастания лесного комплекса Надьэрдё выяснилось, что кислотность почвы является фактором, ограничивающим распространение данного растения. Для установления потребности растения в кислотности, автор применял метод, основывающийся на регулирующей способности корневой системы. Выяснилось, что амплитуда регулирующей способности в небуферной среде изменяется от 3,4 до 9,2 pH, при чем оптимум обнаруживается при pH 5,8. Одновременно произведено изучение почвы следующих, тесно примыкающих ассоциаций:

Festucetum vaginatae danubiale corynephoretosum canescentis,
Festucetum vaginatae salicetosum rosmarinifoliae,
Festucetum vaginatae danubiale.

Определена также буферность и тормозящая способность этих почв. На основании этого подтверждено предположение, что на данной территории указанное растение встречается только на тех местах, где буферность и тормозящая способность почвы минимальная. На почвах с большой буферностью, которую растение уже неспособно урегулировать своей корневой системой, оно уже не произрастает.

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ADAPTATIVE SHIFT OF TISSUE METABOLISM IN LOCAL HYPOXIA RESULTING IN HIGHER MUCOPOLYSACCHARIDE CONTENT

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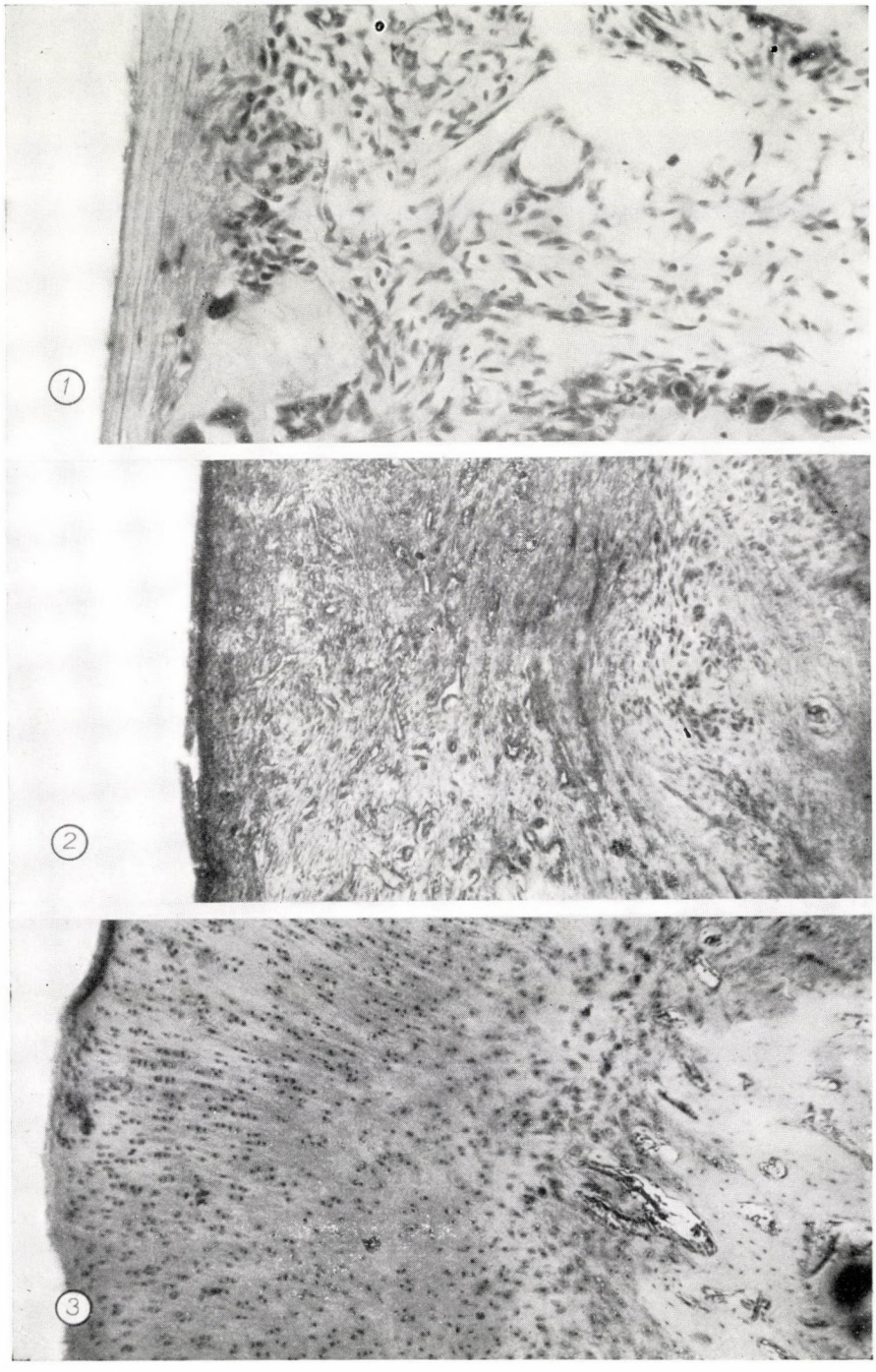
Synopsis

The new formation of articular cartilage from granulation tissue was investigated by morphological, histochemical, biochemical etc. methods. In the initial stage the granulation tissue supplied by capillaries showed a predominant oxybiotic metabolism. Parallel with the gradual occlusion of the capillaries an adaptative shift of tissue metabolism from oxybiosis to anaerobic glycolytic fermentation was observed. Later on, parallel with increasing local oxygen deficiency, the anaerobic glycolysis was found to decrease, simultaneously the mucopolysaccharide content of the tissue increased. The latter process appeared to be parallel with the formation of hyaline cartilage.

Three subsequent maxima occurred. In the stage of granulation tissue the conditions of *oxybiotic metabolism* were given, while the stage of fibrotic transformation of the tissue is characterized by *anaerobic glycolytic metabolism*. In the stage of newly formed hyaline cartilage a further form of metabolism was found to be prevalent, characterized by lack of conditions for oxybiosis, decrease of lactic acid content and increase of *mucopolysaccharides*.

Introduction

The regeneration of the articular surface with special regard to cartilage formation was studied by authors for a considerable period. The morphological, histochemical and biochemical examinations were carried out on an adequate experimental material: on the so-called half-joint of Krompecher [4]. This biological model was studied by Krompecher — chiefly from causal viewpoint of cartilage formation — regarding qualitative adaptation and neodifferentiation [8, 9, 10, 11]. The study of the process of cartilage formation, in particular its metabolic aspects yielded interesting data. It has been established that due to considerable reduction of vascularisation of the articular surface not only respiration decreases but even the glycolytic activity becomes greatly restricted although no signs of necrosis were observed in the tissue. This occurren ce and the remarkable increase of the hexosamine content of the tissue in the regenerating articular surface seem to be interrelated on the basis of the dat a described below in detail.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Material and methods

Investigations were conducted on 450 dogs. On the kneejoints of these dogs the "half-joint" of KROMPECHER was performed. The cartilaginous articular surface of the distal part of the femur was completely removed and subsequently a new articular surface was formed of the granulation tissue originating from the spongious bone.

The dogs were sacrificed after 7, 20, 26, 33 and 70 days following operation (for detailed description of the operation see [9, 10, 11, 4]). On the regenerating articular surface the following morphological and biochemical examinations were carried out: conventional histologic procedures [8, 9, 4], determinations of the degree of vascularisation by planimetry [5], determinations of haemoglobin (WONG) and tissue respiration by WARBURG's method [14], determinations with the same method but after the addition of methylene blue and glucose [6], examinations of anaerobic and aerobic glycolysis according to WARBURG [7], determinations of tissue hexosamine content using the method of MORGAN and ELSON [15].

Results

Study of the newly formed articular surface after different intervals following operation revealed some characteristic stages. On the 7th day the development of a granulation tissue rich in capillaries was observed in the bone wound (Fig. 1). In this stage of development the conditions for oxybiosis are given, *i.e.* the metabolic products of glucose can be broken down through the tricarboxylic acid (SZENT-GYÖRGYI and KREBS) cycle. On the 20th day, in the deepest layer of the granulation tissue, on the margin between the granulation tissue and bone, stenosis of the capillaries occurs. The granulation tissue becomes dense and fibrotic. Subsequently, the capillaries of the deepest layer become occluded [9, 4]. On the 26th day, the stenosis of the capillaries begins to extend and to spread over the median zone. In the depth of the granulation tissue lying directly on the bone border the first cartilaginous formations appear. In a next stage (on about the 33rd day) 3 zones can be distinguished: 1. an *upper zone* with wide capillaries where the conditions for oxybiosis are present; 2. a *median zone* with compressed capillaries where the conditions for oxybiotic metabolism are reduced and anaerobic glycolytic metabolism prevails; and 3. a *lower zone* in close vicinity of the bone where the cartilage islet has developed (Fig. 2). The metabolic conditions of this zone are discussed in the present paper.

Fig. 1. Granulation tissue covering the bone wound after removal of the cartilaginous articular surface. 7 day-stage. Undifferentiated granulation tissue with wide blood vessels. See also next stage

Fig. 2. 26-day stage of the new formation of articular surface from granulation tissue. In the deeper part cartilage islets covered with a perichondrium-like sheet. In the upper layer granulation tissue with patent vessels. At the perichondrium-like border the blood vessels are compressed

Fig. 3. New formation of cartilaginous articular surface. 350 day-stage. The entire granulation tissue has been transformed into cartilage

When studying the process in its development we have to consider that the cartilage of the lower zone increases at the expense of the median and upper zones. The upper zone being at the site of oxybiotic metabolism will be the first to disappear, giving place to the median and subsequently to the cartilaginous lower zone. The cartilaginous islets are still small and isolated on the 33rd day but they gradually get into touch with each other and become

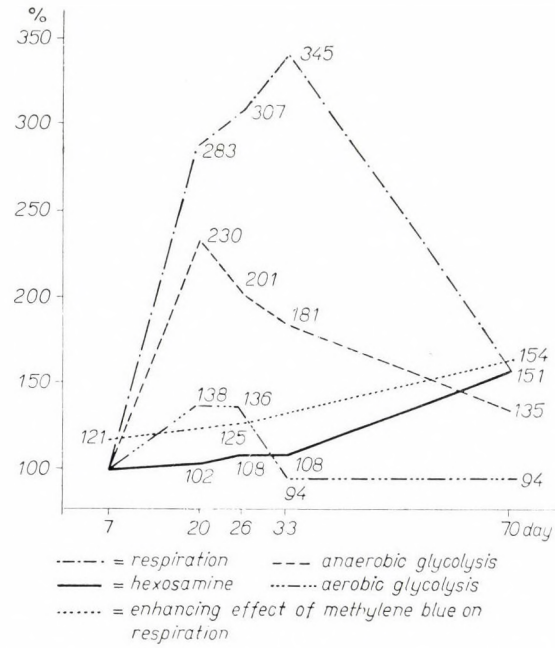

Fig. 4. Graph illustrating the alteration of respiration, anaerobic and aerobic glycolysis, hexosamine content and the enhancing effect of methylene blue on respiration. 7, 20, 26, 33 and 70 day-stage of differentiation of the granulation tissue into cartilage

on about the 70th day almos confluent (Fig. 3). Through the interspaces the vessels maintain the decreasing supply of the granulation tissue of the upper zone.

For complete new formation of cartilage more than a year is necessary.

On the basis of the data obtained from the many-sided investigation of this material the following major conclusions can be drawn.

1. The vascularisation and circulation of the newly formed articular surface respectively, display a gradual decrease. The planimetrically measured cross-section of the capillaries [5] was found to decrease from 6 per cent to about 1 per cent between the 7th and 70th day. During this time the haemoglobin content of the articular surface [13] diminished from the initial 40 γ Fe^{+++}/g granulation tissue to about 13 γ Fe^{+++}/g granulation tissue (Fig. 5).

2. The tissue respiration measured [14] by the method of WARBURG increases from 100 per cent to 345 per cent from the 7th to the 33rd day, then it decreases. On the 70th day it amounts to no more than 151 per cent (Fig. 4).

3. The lactic acid content of the tissue [13] also exhibits an initial increasing trend (7th day 644 mg%, 33rd day 1040 mg%) but it becomes considerably reduced in the later stages (70th day 462 mg%) (Fig. 5).

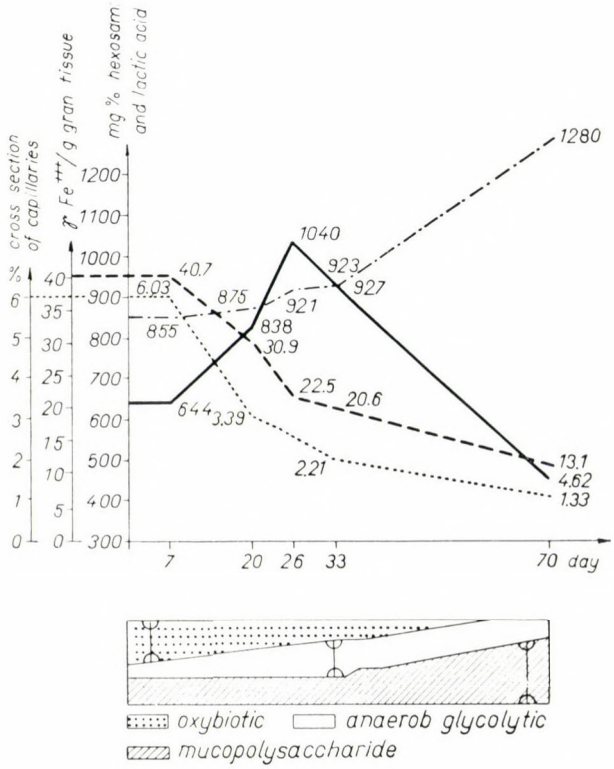

Fig. 5. Graph showing the shifting of metabolism during the development of cartilaginous articular surface from granulation tissue. 7—70 day-stage. Compare the graph with Figs 1, 2, 3. First maximum (lower figure, left side): conditions of oxybiosis in the initial stages are given. Second maximum (middle part of lower figure): the capillaries necessary for oxybiosis are reduced. Lactic acid content reaches its maximum. Cartilage islets appear. Third maximum (lower figure, right side): conditions of oxybiosis (blood supply) no more prevail. Reduction of lactic acid content indicates the decrease of anaerobic glycolysis. Mucopolysaccharide content reaches its maximum

4. Similar data were obtained by determinations of anaerobic glycolysis [7] according to WARBURG's method. The value of 100% anaerobic glycolysis measured on the 7th day rose to 230 per cent on the 20th day after which date it showed a decreasing tendency (70th day: 135%). The maximum value of

aerobic glycolysis was observed [7] on the 20th and 26th day (138 and 176%) subsequently it decreased. On the 70th day the decrease was 94 %.

5. Methylene blue as O_2 transporter, enhanced the respiration rate of the material on the 70th day, while on the 7th and 26th day it was much less effective [6].

6. The tissue hexosamine content on the 70th day showed an increase of 41 per cent (see Fig. 5); increase in the other stages ranged between 2 and 8 per cent [15].

Relative values concerning the results obtained are given in Fig. 4 presenting the data for anaerobic and aerobic glycolysis, tissue respiration and increased respiration caused by methylene blue. It should be noted that these results permit an insight into the respiratory or glycolytic activity respectively, of the tissue in vitro under optimal conditions.

In vivo conditions are more explicitly reflected in Fig. 5. The differences between the in vitro and in vivo states are conspicuous in respiration. While in examinations in vitro the oxygen supply was found satisfactory and unchanged, in the examinations in vivo — on account of the decrease in vascularisation — the respiratory conditions were more and more reduced actually, as shown by the lactic acid values, most probably hypoxydosis ensues. On account of the lack of oxygen and of the possible damage or inactivation of the cytochromes the course of respiration becomes disturbed. The oxygen supply decreases, the tricarboxylic acid cycle and the terminal oxidation are gradually inactivated and therefore fermentation becomes predominant. The course of fermentation, however, is not typical. This observation is supported by the finding that the production of lactic acid of the later stages decreases. At the same time large amounts of mucopolysaccharides determined by the hexosamine content of the tissue appear.

Discussion

These data indicate that tissue metabolism in cartilage formation undergoes several changes. In the initial stage there is respiration as well as fermentation (glycolysis) due to the special nature of the granulation tissue. The mucopolysaccharide (hexosamine) content is relatively low.

In the second phase respiration becomes more and more insignificant and fermentation prevails. The mucopolysaccharide content is still low. In the third phase respiration is reduced to a minimum. Even the lactic acid content of the tissue decreases while the mucopolysaccharide (hexosamine) content increases, reaching now a maximum (see Fig. 5).

In other case of oxygen deficiency (varicose ulcer, necrotic caseous tubercles, C_3H carcinoma and thermal injuries [11, 2]) examinations yielded

comparable results indicating that a similar mechanism may work under these circumstances too.

SZENT-GYÖRGYI [17] claims the possible role of the shift in the balance of oxidation and fermentation as different pathways of energy production. Our experimental results seem to point out that a further type of metabolism involved in the balance has to be reckoned with.

Local metabolism is likely to have certain adaptative possibilities when the circulation is gradually inhibited [7 to 11, 13 to 15]. This adaptative change displays first predominance of oxidation then of fermentation which is followed by a phase in which the production of mucopolysaccharides prevails.

The assumption that a metabolic phase may exist in which mucopolysaccharides are produced, was based on the observation that during hypoxydosis both lactic acid production and content decreased. It seems probable that this phenomenon may be in connexion with mucopolysaccharide formation. This supposition has been confirmed by the data of LELOIR and CARDINI [12], CASTELLANI and ZAMBOTTI [3], OHKUBO, NOWINSKI and BLOCKER [16] and of others. According to these data certain intermediaries that take part in fermentation may serve as sources for the formation of the components of mucopolysaccharides. BANGA [1] pointed out that various mucopolysaccharides possess the capacity of inhibiting or stimulating the activity of certain enzymes. It is possible that in the course of mucopolysaccharide formation, in a later stage this effect is also instrumental.

The present publication should give for the time being a working hypothesis for the study of the enzymes and intermediaries of fermentation under the given circumstances. Investigations in this direction are in progress.

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DIE ADAPTATIVE VERSCHIEBUNG DES GEWEBLICHEN STOFFWECHSELS BEI LOKALER HYPOXIE EINHERGEHEND MIT EINER STEIGERUNG DES MUCOPOLYSACCHARIDGEHALTES

Der Vorgang der Neubildung einer knorpeligen Gelenkfläche, ausgehend vom Granulationsgewebe, wurde durch morphologische, histochemische, biochemische etc. Methoden untersucht. Im Anfangsstadium zeigt das gut vascularisierte Granulationsgewebe einen vorwiegend oxybiotischen Stoffwechsel. Parallel mit der stufenweise erfolgenden Kompression der Kapillaren verschiebt sich der gewebliche Stoffwechsel adaptativ von der Oxybiose in Richtung des anaerob glykolytischen Stoffwechsels. Später, parallel mit der Steigerung des lokalen Oxigenmangels, zeigte sich eine Abnahme der anaeroben Glykolyse; gleichzeitig zeigte sich eine Zunahme des stofflichen Mucopolysaccharidgehaltes. Diese Steigerung lief parallel mit der Knorpelbildung.

Drei aufeinander folgende Maxima waren zu finden. Das Maximum der Bedingungen des *oxybiotischen Stoffwechsels* im Stadium des Granulationsgewebes. Darauf folgend, im Stadium der faserigen Transformation des Gewebes, zeigt der *anaerob glykolytische Stoffwechsel* das zweite Maximum. Im Stadium des neugebildeten Hyalinknorpels gelangte eine weitere Form des Stoffwechsels in den Vordergrund, charakterisiert durch das Fehlen der Bedingungen der Oxybiose, eine Abnahme des Milchsäuregehaltes und durch die Zunahme des *Mucopolysaccharidgehaltes.*

АДАПТАТИВНОЕ ИЗМЕНЕНИЕ ТКАНЕВОГО ОБМЕНА ПРИ МЕСТНОЙ ГИПОКСИИ, ПРОВОДЯЩЕЕ К ПОВЫШЕННОМУ СОДЕРЖАНИЮ МУКОПОЛИСАХАРИДОВ

Авторы изучали образование суставного хряща из грануляционной ткани при помощи морфологических, гистохимических, биологических и других методов. В начальной стадии рубцовая ткань, богатая капиллярной сетью, имеет преобладающий, оксибиотический обмен. Паралельно сдавлению капилляров наблюдался изменение обмена тканей адаптативного характера, из ранее выраженного оксибиотического обмена в направление анаэробного гликолитического обмена. Позже, при усилении местного недостатка кислорода, и анаэробный гликолиз отступал на второй план, в то же время увеличивалось и содержание мукополисахаридов в тканях. Последний процесс был параллельно образованию гиалинового хряща, содержащего хондроитинсульфат.

Отмечалось три максимума, следующих друг за другом. В начальной грануляционной стадии имеются условия *оксибиотического обмена;* стадия перестройки волокон характеризуется *анаэробным гликолизом,* а в стадии новообразованного гиалиного хряща появляется новый, дальнейший тип обмена, который отличается следующим: отсутствием оксибиоза, уменьшением содержания молочной кислоты и *увеличением содержания мукополисахаридов.*

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RECENSIONES

CAVENESS, W. C.: **Atlas of electroencephalography in the developing monkey**

Pergamon Press, London (1962), 146 pages. £ 6/6

Introduction to the Atlas has been supplied by HALLOWEL DAVIS, one of the American pioneers of electroencephalography. The author, a member of the Institute of Neurology, Columbia University, in his Atlas traces the changes in the EEG activity of the developing monkey *(Macaca mulatta)* from birth till 24 months of age, i.e. until full maturity. The 96 full-page figures have been selected from among 434 EEG recording in 47 animals. After a discussion of the properties of the strain of monkey used, in Chapters 1 and 2 the technique of the leads is described in detail. Three pairs of needle electrodes were placed subcutaneously in the frontal, temporal and occipital areas, to carry out bipolar longitudinal and transversal leads.

In Chapters 4 and 5 the development of the spontaneous waking activity at rest is dealt with. The forms of activity characteristic of the different ages are illustrated by 35 Figures, with 16 other recordings showing the variations deviating from the average. Subsequently the average amplitude and frequency values of the characteristic alert rhythms are evaluated statistically and represented in tabulated and diagrammatic form. The recordings obtained in the monkey are carefully compared with the data published in the literature for man. The similarity is remarkable, when assuming the rate of development of *Macaca mulatta* to be sixfold that of the human brain, and very explicit as regards the frequency domains. In early age the amplitudes are greater in the monkey, but this difference disappears in adulthood.

In separate chapters, illustrated with 45 figures the transition from alertness to sleep, the EEG characteristics of superficial and deep sleep in the developing animals are discussed. Here, too, a comparison with human activity is given at the end of each chapter, corroborating the view that EEG activity of the developing *Macaca mulatta* shows but a minimum difference as against electrical activity of the human brain of comparable age. It was only in the adult monkey that the cyclic, low-amplitude, fast EEG activity described by DEMENT and KLEITMANN in man could be observed during sleep. Unfortunately, there is no figure to illustrate this, although this observation is a most valuable contribution to the explanation of the physiological mechanism of these cycles. In the Appendix the curves characteristic of the various ages are presented comprehensively. The material included in that chapter is particularly well selected.

In the past decade EEG has become an everyday tool both in therapeutical and experimental medicine. One of the most difficult problems in clinical electroencephalography is the analysis of the EEG activity in children. Along with the works dealing with the electrical activity of the developing human brain this Atlas may offer valuable help for clinicians concerned with the EEG of children.

Rhesus monkeys are being used more and more frequently as test subjects in neurophysiological and psychopharmacological studies. The use of these animals makes it possible for the research workers to work on preparations resembling the human brain in function and structure much closer than the brain of any other species generally made use of before. Research workers unable to deal with large series because of the high price and insufficient availability of monkeys who therefore could not gain personal experience in the field of monkey EEG may derive most valuable information from this excellently composed and well arranged Atlas and may take into account the special data characteristic of the various ages when working with younger animals.

The Atlas is beautifully executed; the excellent reproduction of the records should be particularly stressed.

KÁLMÁN LISSÁK (Pécs)

SIEGEL S. M.: **The Plant Cell Wall.** A Topical Study of Architecture, Dynamics, Comparative Chemistry and Technology in a Biological System

Pergamon Press, Oxford (1962), 123 pp., 60 s.

Although this little book by S. M. SIEGEL gives a brief outline of the vast subject in all its more significant aspects, it is mainly from a chemical, respectively biochemical perspective that the author approaches the problems involved. The book opens with an introduction briefly describing the constitutional features of the cell wall recognizable in the light-microscope and the cell types of the most frequent occurrence (parenchyma, sclerenchyma, collenchyma, tracheids, vessels, fibres, etc.). This is followed by a chapter on "Constitution and Architecture in the Cell Wall", beginning with the examination of some of the chemical-physical methods employed in studies on the chemical constitution and structure of the cell wall (polarized light, X-ray diffraction, ultracentrifuge, etc.), after which the results obtained with the aid of these techniques are dealt with. In this connection, the author gives much attention to the organization of the polysaccharides (cellulose, pectin, etc.) constituting the cell wall and to that of lignin. Under the heading "The Wall as a Unit" consideration is centered about the crystal lattice of cellulose, the structure of elementary fibrils, microfibrils and the orientation of cellulose microfibrils in the cell wall, respectively in its different layers. In Chapter 2 (Cell Wall Dynamics) a brief, but particularly interesting description is given of current knowledge as to the biosynthesis of cell wall components, first of all, of the mechanism governing extracellular cellulose formation *(Acetobacter xylinum)*. Novel and important problems are touched upon in the pages where the changes occurring in the walls of growing cells are discussed, — mainly from biochemical viewpoints, — and further, where the regulation of cell wall formation is considered in connection with the effects of growth-promoting substances (auxins) and lignification. A short sub-chapter is dedicated to the various possibilities of natural cell wall breakdown (The Lysis of Cell Walls). In Chapter 3 (Comparative Chemistry of Intercellular Substances and Walls) the occurrence and biosynthesis of lignin in higher vascular plants, as well as the cell wall chemistry of bacteria, fungi, algae, protozoa and metazoa are examined, with respect to their evolutionary significance and their phylogenetic aspects. In Chapter 4 which is the last one (Properties and Uses of Cell Walls and their Derivatives) the practical aspects of the subject are presented, the author giving a brief account of the chemical, physical and structural properties of woods and fibers with a view to their industrial uses. After discussing the coal, its origins and the process of coalification the book closes with a list of references, an index of the organisms dealt with in the text and a general subject index.

LORÁND FRIDVALSZKY (Budapest)

DOUGHERTY, T. F., W. S. S. JEE, CH. W. MAYS, B. J. STOVER: **Some Aspects of Internal Irradiation.** Proceedings of a Symposium held at the Homestead, Heber, Utah, 8—11 May, 1961

Pergamon Press, Oxford (1962), 529 pp., 69 tables, 242 ill. £ 5

The bulky volume in a fine get-up, published by the Symposium Publications Division of Pergamon Press, comprises the lectures and the discussion at the Symposium held in Heber (Utah). The Symposium was concerned with the harmful effects of radioactive substances got into and incorporated by the organism as well as with those of chronic low-level radiation. The ample material, the 33 lectures delivered, are grouped into 5 parts by the editors.

Part I, containing 11 lectures discussing the various biological effects of internal irradiation, is the most comprehensive of all. All the lectures render account of the researches conducted at the University of Utah, College of Medicine, Salt-Lake City. The experiments referred to in the lectures were carried out on dogs, on beagles. Ra^{226}, Ra^{228}, Th^{228}, Pu^{239}, Sr^{90} were applied at different dose levels, as internal emitters. The distribution of the said radionuclides in the organism as well as the long-term biological effects of chronic radiation were studied. Because of the bone-seeking character of the applied radionuclides, to bone injuries and to the development of bone tumours were given the greatest attention. However, one of the lectures discusses the hematological damages, another the changes occurring in blood chemism, a further one the eye changes induced by internal irradiation. Finally, a lecture deals with the effect of radionuclides on corticosteroid biosynthesis.

Part II comprises 8 lectures, discussing the biological effects both of internal irradiation and X-ray irradiation. Two communications deal with the problems of low-level irradiation in general, respectively, with the reparable and irreparable properties of radiation injury. An interesting lecture reports on the role the hormones play in radiation-carcinogenesis. Two papers deal with the changes in premature — generalized and localized — radiologic aging, a further one discusses the effects of continuous irradiation at different dose-rates on the hematopoietic organs. Another lecture is consacrated to the specific radiation injuries of the lung and one to the radiation effect on the bone-forming cells.

The five lectures forming Part III discuss some problems of the toxicity of Sr^{90}. The harmful effects of the prolonged administration of Sr^{90} — in the first place on the bone structure and on the bone marrow as a hematopoietic organ — were studied on monkies, beagles, rabbits, rats and Pitman—Moore miniature swine. This latter species is considered as extremely suitable for experimental purposes, since the animals weigh 60—70 kgs. when mature, are omnivores and their gastro-intestinal tract and bone mass are most similar to those of man.

Part IV includes the 6 lectures delivered on the dosimetry of internally deposited radionuclides. One of these lectures discusses the interaction of alpha and beta particles with matter, another one the relationship between alpha and beta dosimetry. The remaining lectures approach the difficult task of *in vivo* dosimetry, both theoretically and experimentally. One of the lectures reports on the skeletal retention of alkaline earth radioisotopes.

Part V contains but two lectures discussing the delayed harmful effects of Thorotrast (thorium dioxide), formerly applied in diagnostic medicine. One of these communications reports on 842 individuals who had been given Thorotrast injections and many of whom developed malignant tumorous diseases in course of the subsequent years. The other lecture treats of the metabolism of thorium daughters.

Thus, the volume publishes quite a number of more recent data concerning the results of researches in the field of toxicology of radioactive substances. The production of nuclear fission materials and the more and more extensive application of radioactive isotopes rendered the researches in the field of radiotoxicology continuously more important. The communications in this volume summarize the results of generally thorough and detailed researches. It is of importance to point out that the experiments on dogs were always carried out on individuals from the very same strain.

It would be a difficult task, indeed, to prefer any part of the book to the others. Our stating that the communications in Part II and the lectures on the dosimetry of incorporated radioactive substances are of a special interest to us, represents by no means a grading. The publication of the sometimes rather active and thorough discussions following each lecture renders the book even more precious to the specialist. The University of Utah College of Medicine undertook a most important task, indeed, when organizing this Symposium and by publishing the written record of the same, Messrs. Pergamon Press promoted the development of radiotoxicology a great deal.

78 specialists, engaged for a long period of time in investigating the biological effects of radionuclides got into the human and animal organism — many of whom are the most outstanding experts on this problem — attended the Symposium. This enabled the manysided discussion of the data presented. In the literature of radiobiology disproportionately more thought is given to the effects of external radiations and the number of International Symposia discussing the pathological effects of internal emitters is insignificant. Also, there are but a few general works on radiotoxicology. This is also one of the reasons one is pleased to have this volume published. We do hope that similar works will follow in the future.

Printing was done in Poland, Warsawa. Apart from the relatively small number of errata, the typographic execution in general equals the contents. Though the quality of the photos is objectionable, even the microphotographs can be satisfactorily interpreted. The other illustrations and the graphs are perfect.

VILMOS VÁRTERÉSZ (Budapest)

Printed in Hungary

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ELECTRON MICROSCOPY OF PARATHYROID SECRETION IN RANA ESCULENTA

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DEPARTMENT OF PHYSIOLOGY AND BIOLOGY, MEDICAL UNIVERSITY, PÉCS (HEAD: K. LISSÁK)

(Received June 20, 1962)

Synopsis

Parathyroid hyperfunction was induced in frog by experimental hypocalcaemia due to EDTA-Na_2 administration and hypofunction was evoked by hypercalcaemia due to AT-10 and $CaCl_2$ administration. Changes in different organelles were traced in electron microscope studies. Observations demonstrate the secretory process to be the result of a complex nucleo-cytoplasmic interaction. Authors establish the following assumptions concerning the role played by individual organelles in the secretory process. The Golgi apparatus has an indirect role by producing empty vesicles. Vesicles leaving the Golgi ground substance are filled in the cytoplasm either with the specific secretory substance or with its precursor and thus become transformed into secretory granules with great density and increased diameter.

Introduction

Submicroscopic study of secretory processes in endocrine glands has no long history and left a fair number of problems still unsettled.

This is especially true for the parathyroid gland. Light microscopic studies were performed with mainly morphological targets. In certain papers, however, the problem of the secretion mechanism has been already raised. Light microscopic descriptions of the finer mechanism of parathyroid secretion are not fully reliable and often contradictory data are found in papers by different authors.

Rosof [25] performed a study on rat parathyroid and arrived at the conclusion that changes in the cytoplasm due to different secretory phases are reflected in the morphological appearance of the individual cells. On this basis light and dark cells may be distinguished. According to this author a certain substance, identical with the Golgi-apparatus-mitochondrial complex producing the secrete, can be found in the cytoplasm.

The cytology of rat parathyroid is not difficult to survey since only light and dark cells were found in this species by all workers. Castlemann and Mallory [3] confirmed this finding by demonstrating the absence in rats of oxyphil cells characteristic of ungulates and primates. De Robertis [6] found a relationship between the normal or increased function of the gland and the cell types. In hyperfunction, an increased number of dark cells occurred. Bensley [1] regards the appearance of dark cells as being due to a certain

physiological state of secreting cells which is characterized by high lipid and low water content of the cytoplasm. PAPPENHEIMER [21] ascribes a role in secretion to the basophil or iuxtanuclear structures. In variance with the above views COWDRY [4] questions any dependency of the appearance of cytoplasm on the stages of secretion. The above survey of previous works makes it clear that light microscopy only slightly promoted the understanding of the mode of secretion and especially of the finer mechanism of this process.

In recent years electron microscopy in more than one respect approached the general problem of the secretory process but the main emphasis was laid on the exocrine glands. Less is known concerning the endocrine glands and particularly as to the parathyroid.

LEVER [13] performed a detailed electron microscopic study on rat parathyroid and rejected the static distinction of dark and light cells, stating that a single cell type may appear in different — light or dark — cell forms. In a subsequent publication [14] the same author reported on the submicroscopy of the secretion demonstrating the dependence of the state of cytoplasm on the secretory process and dominance of dark cells in hyperfunction. He verified his earlier findings on mutual transformation of light and dark cells. The ergastoplasm is regarded as the site of secrete production as shown by its dilatation in hyperfunction and slackening in hypofunction. Increased secretion is accompanied by an increase in the number of ribosomes.

STOLL's [29] results on thyroid gland also led to the assumption of ergastoplasmic origin of the colloid.

The experimental results of LEVER were apparently invalidated by a communication of EKHOLM [7] stating that erroneously thyroid gland instead of parathyroid was used by the former author, while LEVER [15] assumes that adipose tissue was mistaken for parathyroid by EKHOLM.

In the present work frog parathyroids were studied, as only a single cell type has been found in this gland in light microscopic studies [24, 31]. The ultrastructure of the organ was described in an earlier study [16] which also revealed the presence of only a single cell type.

Experimental hyper- and hypofunction of parathyroid was induced to obtain further information on the role in secretion of individual organelles.

Materials and methods

Female frogs *(Rana esculenta)* of 100 g weight were used in the present experiments performed in early spring in order to avoid seasonal influences. The glands were extirpated in ether narcosis according to the — somewhat modified — method of BOMSKOW [2] and immediately fixed in PALADE's osmic acid for 15 min. at room temperature [17]. The material was embedded in an 1:4 mixture of methyl and butyl methacrylate. The mixture was prepolymerized to a honeylike consistency. 300 to 500 Å thick sections were cut on an LKB Ultrotome and examined with a Zeiss ELMI D 2 electron microscope.

Hyperfunction of the gland was induced by experimental hypocalcaemia. This condition was obtained by s. c. injection of aqueous solution of ethylendiamine-tetraacetic acid (dinatrium

salt — K. III) in an amount of 20 mg per 100 g body weight. This amount was given twice daily for 6 days.

Hypofunction was induced by experimental hypercalcaemia. Dihydrotachysterine (AT-10) and calcium chloride were administered through a gastric sound. 0.5 ml AT-10 and 0.5 ml 10 per cent $CaCl_2$ were given twice daily for 6 days.

Not all animals were subjected to a morphological study. In most of them only the blood and urine Ca-level was examined to reveal their changes. The serum Ca-level was determined immediately after the extirpation in animals whose glands were embedded for electron microscopy. The Ca-levels were established according to a complexometric method with eriochrome indicator [10].

Glands of hyper- and hypocalcaemic animals were fixed in the same fluid and at the same time and embedded into the same medium to minimize errors due to microtechnical treatment.

Results

The first experiments were intended to demonstrate possible active Ca-regulation in the frog, *Rana esculenta*. We present now results which pointed to the development of hyperfunction when artificial hypocalcaemia was induced by the method referred to above. Table 1 shows the effect of prolonged K. III treatment against the normal original Ca-levels. This treatment reduced the mean value of serum Ca from 11.3 mg to 8.3 mg per 100 ml and increased the urine Ca from 1.3 mg to 4.3 mg per 100 ml. These values show that actually hypocalcaemia was produced. The increase of urine Ca-level can be most probably explained by renal excretion of Ca bound in the serum by K. III and by its release in the urine.

Table 1

Effect of K. III treatment (for 6 days) on serum and urine Ca-level (mg per 100 ml) in Rana esculenta

No of animal	Normofunction		Hypocalcaemia	
	serum	urine	serum	urine
1	11.0	1.7	8.5	4.7
2	11.3	1.5	8.2	4.5
3	11.5	1.1	8.3	2.7
4	12.0	1.0	8.0	4.6
5	11.0	1.7	8.4	4.8
6	11.2	1.8	8.7	4.5
7	11.4	1.5	8.5	4.9
8	11.3	1.2	8.9	4.1
9	11.0	1.3	8.0	4.5
10	11.6	1.0	7.8	4.6
mean	11.3	1.3	8.3	4.3

In some animals hourly determinations of serum and urine Ca-level were made after K. III administration. Fig. 1 shows that the decrease of serum Ca-level evoked by K. III is only temporary. One hour after treatment the level is already rising and becomes almost normal after 6 hours. A complete restoration, *i.e.* a rise over 11 mg per 100 ml is seen after 18 hours. Control of K. III effect by the frog points to increased parathyroid activity. If this conclusion is valid, there is an active Ca-regulation in amphibia.

Changes in urine Ca-level follow an opposite trend the possible explanation of which was already exposed.

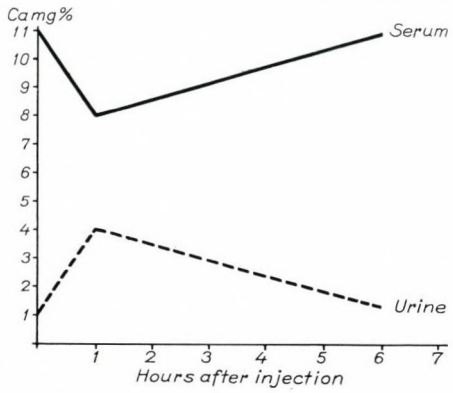
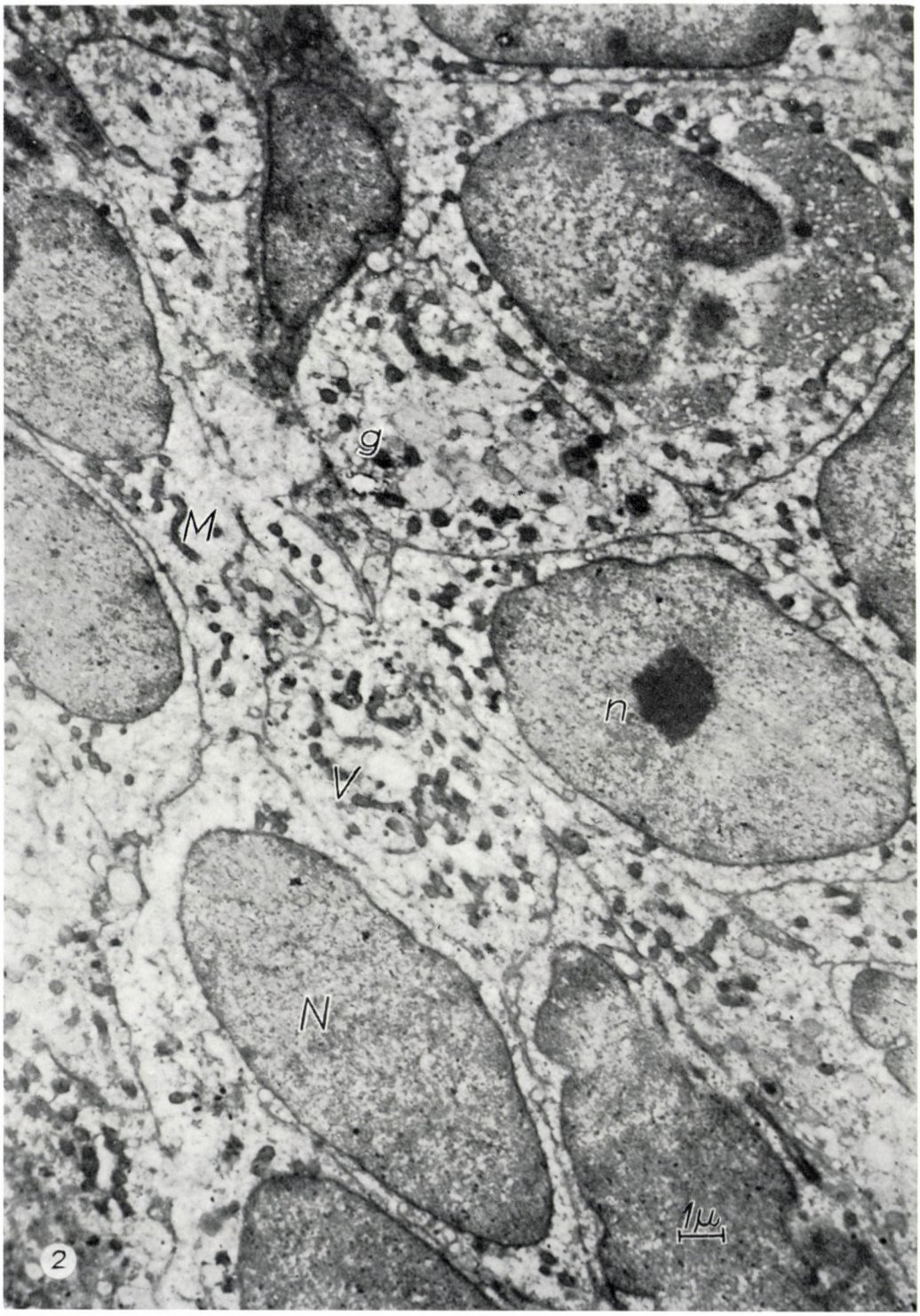


Fig. 1. Changes of serum and urine Ca-level in a frog following s. c. injection of EDTA—Na (K. III)

These preliminary experiments allow the assumption that hypocalcaemia due to prolonged K. III treatment induces a hyperfunction of the parathyroid. The electron microscope study of glands in hyperfunction may contribute to the knowledge of the mode of parathyroid secretion by revealing those organelles which exhibit the greatest changes as compared with the normal situation.

In the cytoplasm of hyperfunctional cells secretory granules of great density and increased diameter are prominent. In normal glands these granules have a diameter of 700 mμ whereas in hyperfunction they attain 100 to 1200 mμ (Figs. 6 and 7). In hyperfunctional cells allmost all secretory granules are filled with a very dense substance and the membrane-bound granules of low density which are so frequently found in normal glands are almost missing (Figs. 2 and 3). A further difference between the two granules appears in their

Fig. 2. Low-power view of frog parathyroid in normofunction. The mass of the cytoplasm is small compared to the large nucleus (N) which usually contains one nucleolus (n). Numerous mitochondria (M) few secretory vesicles (V) and slightly dense secretory granules (g) are seen in the cytoplasm. × 5200


g
M
n
V
N
1μ
2

membrane which is continuous in the normal glands but shows discontinuities in hyperfunction. Electron micrographs show the leakage of the secrete through the discontinuities into the cytoplasm (Figs. 4 and 6). Certain granules have retained only small portions of their membrane and convey the impression as if their content were eluated in the cytoplasm (Fig. 8). This mode of release of the secretory granule content is rarely seen in normofunction (Fig. 6).

Mitochondria are shorter and have an intensely osmiophil membrane in hyperfunction (Figs. 3 and 8).

The Golgi apparatus is more pronounced in hyperfunction. Its ground substance includes numerous empty vesicles. No structures, osmiophil substance or electron density are found in these vesicles. Larger vacuoles move to the periferic zone of the apparatus and migrate into the cytoplasm (Figs. 5 and 8).

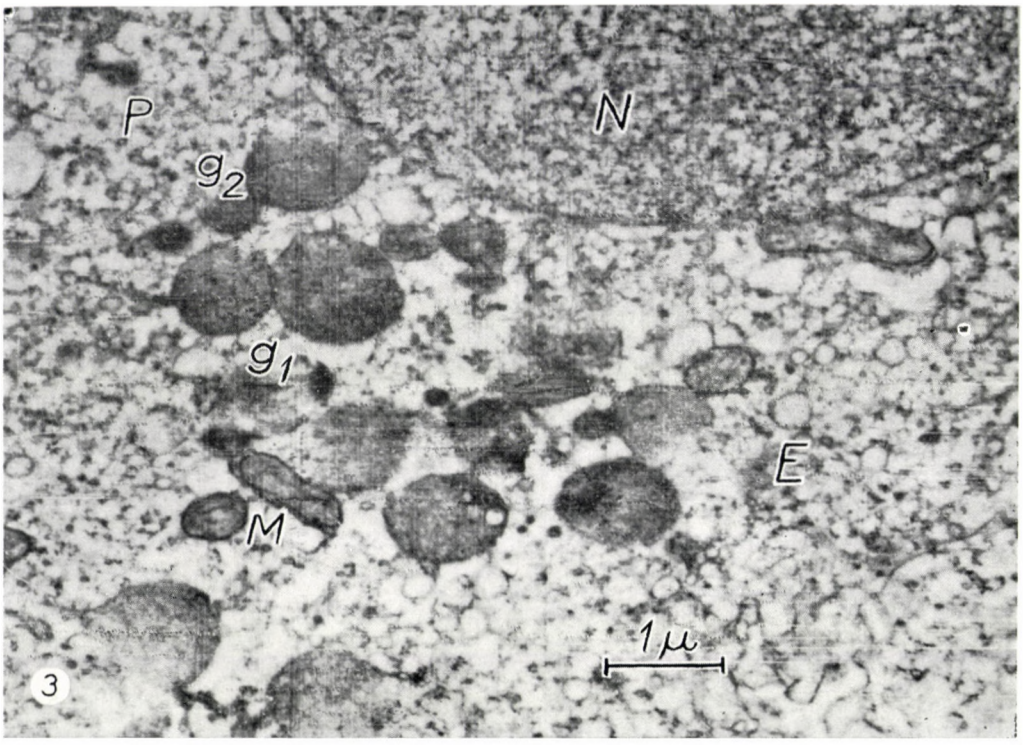
In the nuclei following changes are observed and thought characteristic of parathyroid cells in hyperfunction. The nuclear membrane has larger and more frequent pores than in normal cells. Structures of the karyoplasm become enhanced as expressed by the appearance of channels which run from the pores into the karyoplasm. Isolated areas were also seen in the karyoplasm in certain cases. WETZSTEIN [33] found similar areas in adrenal medulla.

In the last series of experiments frog parathyroids were studied in hypofunction evoked by artificial hypercalcaemia as described above.

Table 2 shows that AT—10 and $CaCl_2$ treatment induced a marked hypercalcaemia. The mean serum Ca-level rose from 11.3 mg to 13.5 mg per 100 ml. Urine Ca-level also increased, from 1.3 mg to 9.4 mg per 100 ml. The latter effect finds its explanation in the probable absence of AT—10 effect on intestinal Ca-resorption in amphibia. AT—10 never induces as high Ca-levels in amphibia as in mammals. In our unpublished experiments much higher Ca-levels were obtained in rats by means of prolonged AT—10 treatment.

The ultrastructure of parathyroids in hyperfunction differs to a great extent from that of normal or hyperfunctional glands. Secretory granules are rarely seen in the cytoplasm. Mitochondria and ergastoplasm are largely destroyed. No nuclear pores are found and the usually finely homogeneous nuclear structure shows a coarse precipitation (Fig. 9).

Fig. 3. Cytoplasmic area of hyperfunctional frog parathyroid. Secretory granules are very dense (g_1.). In certain granules (g_2) the membrane is dissolved and the content released into the cytoplasm. Mitochondria (M) are bound by thick osmiophil membrane. The cells in hyperfunction are characterized by numerous Palade granules (P) and by the marked development of the saccular ergastoplasm (E). In the upper part a nucleus is seen (N). ×15000. *Fig. 4.* Part of a hyperfunctional cell. All secretory granules (g) are very dense and release their content into the cytoplasm. × 16500. *Fig. 5.* Golgi apparatus of a cell in hyperfunction. In the lamellar ground substance (1) of the apparatus Golgi vesicles (V) in formation. The size of the vesicles increases as they approach the periphery. × 24000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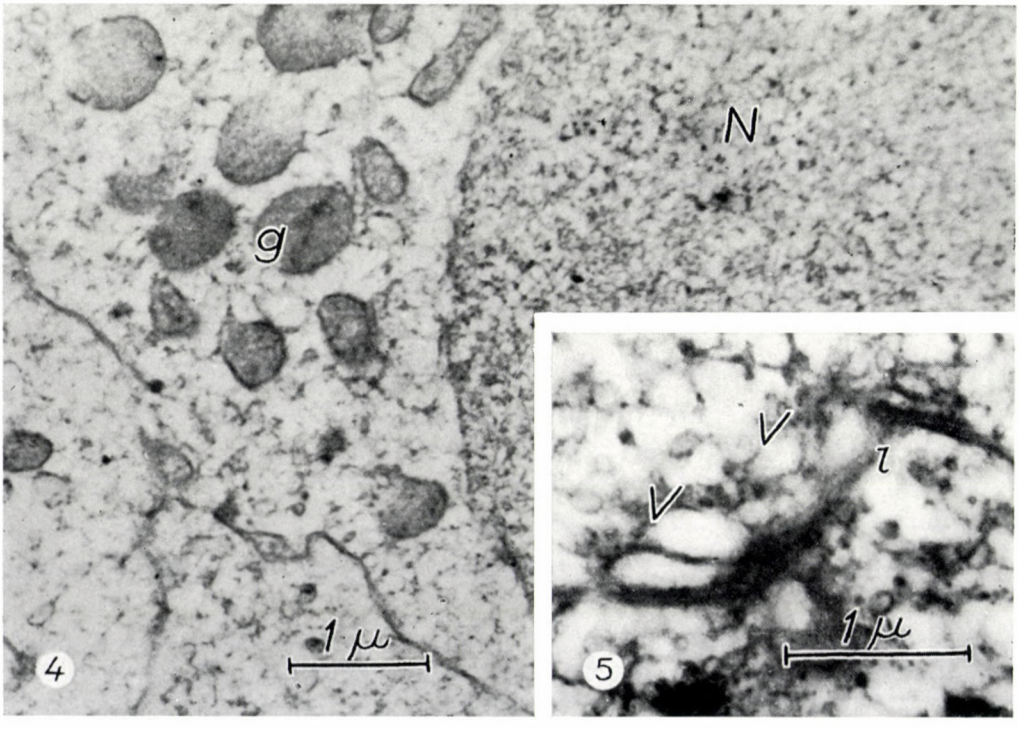
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Table 2

Effect of AT—10 *and calcium chloride treatment (for 6 days) on serum and urine Ca-level (mg per 100 ml) in* Rana esculenta

No of animal	Normofunction		Hypercalcaemia	
	serum	urine	serum	urine
1	11.0	1.7	13.2	7.0
2	11.3	1.5	14.1	13.1
3	11.5	1.1	13.5	12.5
4	12.0	1.0	13.1	10.4
5	11.0	1.7	13.4	6.5
6	11.2	1.8	13.1	13.5
7	11.4	1.5	13.6	9.4
8	11.3	1.2	14.0	7.2
9	11.0	1.3	13.5	8.4
10	11.6	1.0	13.7	9.3
mean	11.3	1.3	13.5	9.4

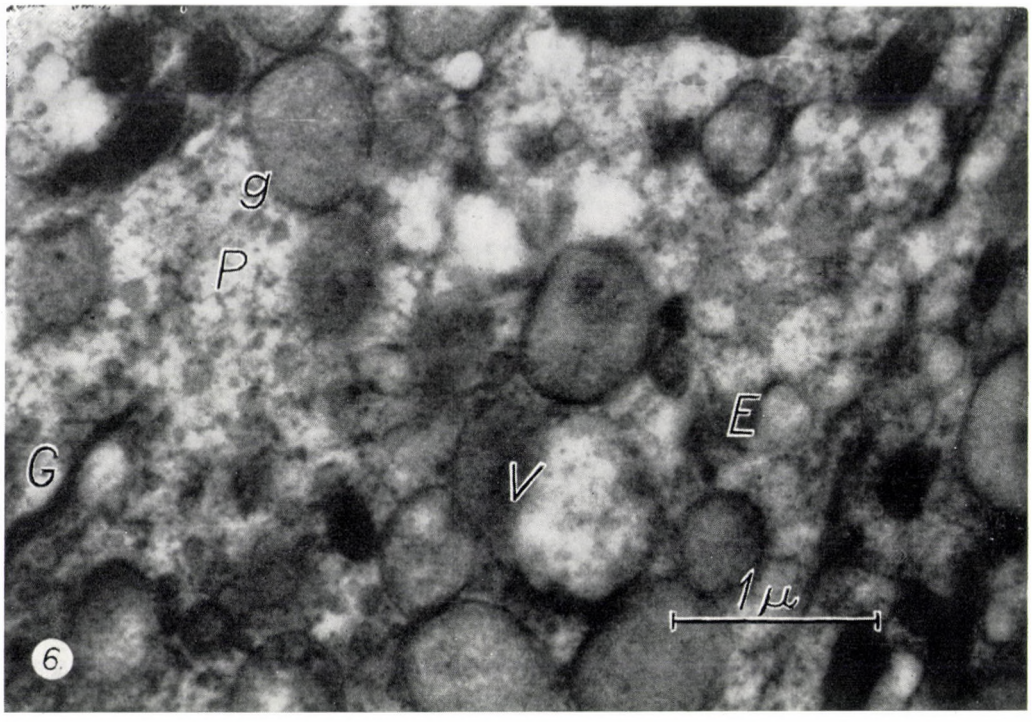
Discussion

According to the results reported in a previous [16] and in the present paper marked differences can be observed in secretory granules, Golgi apparatus, mitochondria, ergastoplasm and nuclei of frog parathyroids in normal, hypo- or hyperfunctional state. This points to a role of these structures in the secretion of parathormone.

Numerous contributions demonstrated the protein synthesis, *i.e.* in this case the formation of the secretory substance to proceed in the basophilic parts of the cytoplasm, *i.e.* in the cisternes and vesicles of the ergastoplasm. This view is supported among others by PALADE [18 to 20] and PORTER [22]. DEMPSEY [5] found that the colloid forms and accumulates in the saccules of the thyroid ergastoplasm.

In hyperfunctional rat parathyroids LEVER [14] found a dilatation of certain parts of the ergastoplasm. This finding is in accordance with the views of the authors referred to. No similar changes were observed during the present study in frog. In the case of hyperfunction most marked changes were found in the secretory granules. TRIER [30] reports membrane-bound granules from parathyroids of primates, which were suggestive of the granules observed by us.

Fig. 6. Cytoplasmic area of a cell in normofunction. Empty secretory vesicles (V) and numerous semiopaque secretory granules (g) of 700 mμ diameter are seen. Golgi apparatus (G), Palade granules (P) and vesicular ergastoplasm (E) are also shown. × 26400. *Fig. 7.* Cytoplasmic area of cell in hyperfunction. Secretory granules (g) are increased to a diameter of 1200 mμ. × 26400


g
P
G
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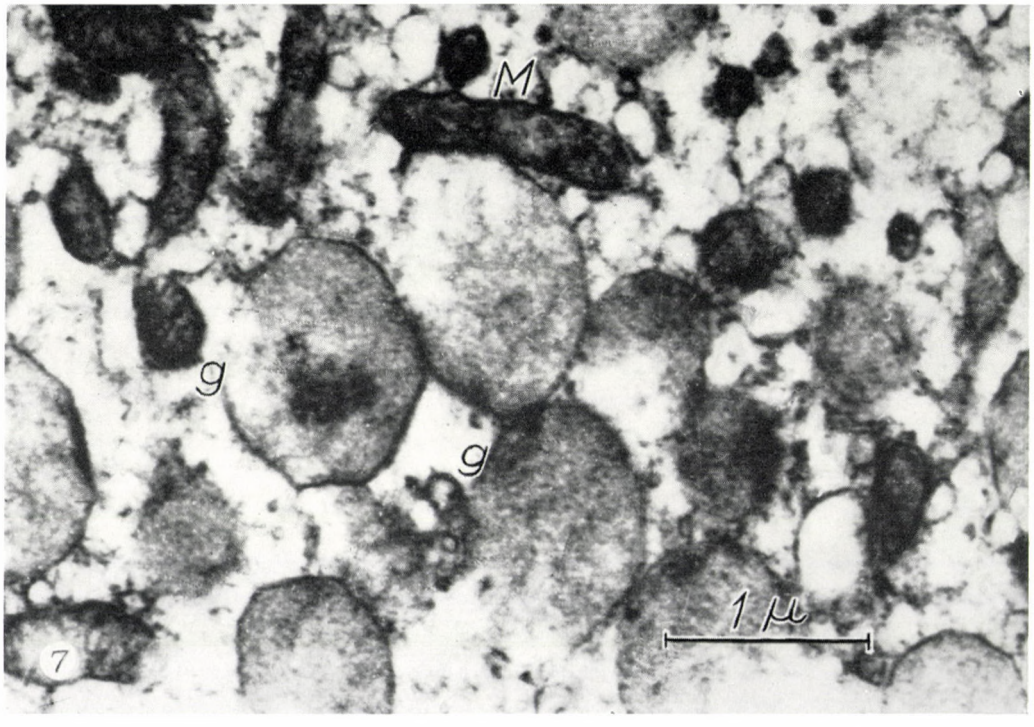
6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The hyperfunction, *i.e.* enhanced hormone formation in frog parathyroids is accompanied by enlargement and increased density of the secretory granules. On these grounds it may be assumed that these granules carry the parathormone or its precursor. According to literary data outlined above the ergastoplasm is mainly involved in the formation of the secretory product. In hyperfunction, an increase of the number of the Palade granules was observed also in the present study.

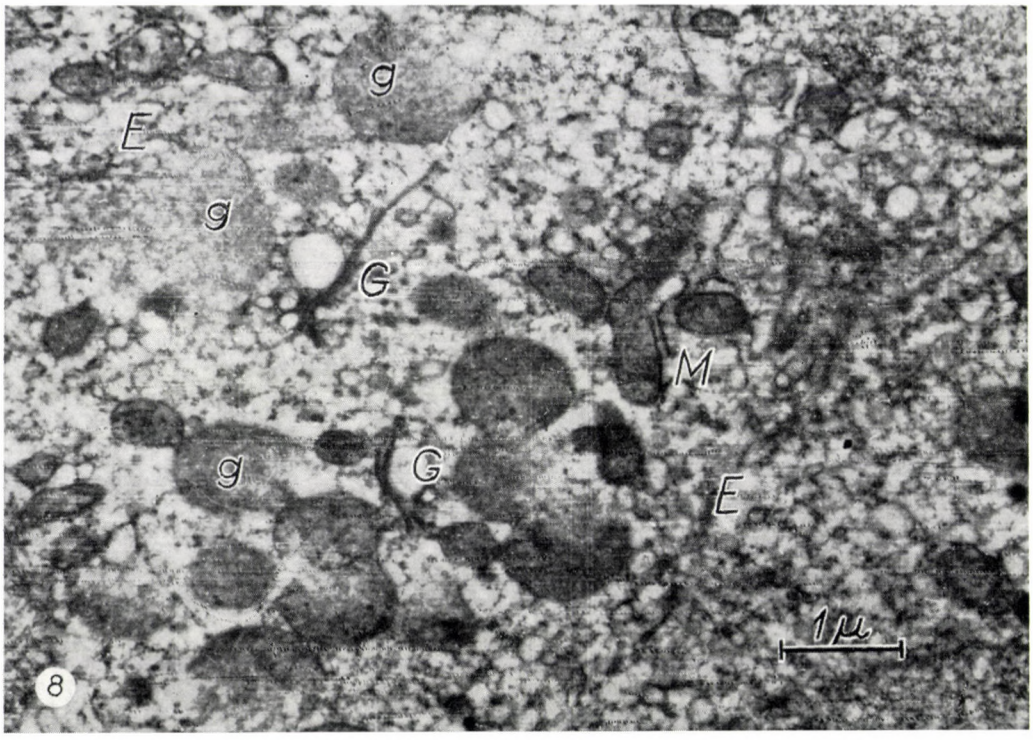
The next two issues of the mode of inner secretion are represented by the genesis of the secretory granules and by the release of the secrete by the cells.

As to the genesis of the secretory granules certain clues might be obtained from the study of the Golgi apparatus. Our experimental results allow the conclusion that in the Golgi ground substance empty vesicles are formed which pass into the cytoplasm and get filled there with the secretory product. This assumption is supported by observations made by FARQUHAR and RINEHART [8, 9], GOODSIR [11] and SEVERINGHAUS [26]. RINEHART and FARQUHAR [23] found intimate interaction between Golgi substance and the granules in the chromophobe cells of the anterior hypophysis. They found undeveloped Golgi substance when no granules were present.

In variance with the opinion of SCHNEIDER [27, 28] who assumes that the definite specific products are formed in the Golgi substance, the present observations do not permit a similar conclusion. We observed larger secretory granules which, especially in the case of hyperfunction, contain a large amount of osmiophil substance whereas the vesicles resp. vacuoles in the Golgi substance are smaller and empty. It may be assumed, therefore, that the role of the Golgi apparatus is to supply empty vesicles.

The assumption that the Golgi system produces only the membranes of the secretory vesicles while it is not involved in the synthesis of the specific cell product, is supported to a great extent by literary data concerning the chemical composition of the Golgi apparatus. BAKER [12] demonstrated the presence of lecithine, kephaline and sphyngomyeline by histochemical methods. The predominance of lipid components was supported by the observations of GRANAGLIA [12] and BERG [12] on the failure of the Aoyama impregnation to demonstrate the Golgi apparatus in pancreas cells treated with lipid solvents. GERSH [12], ARZAC [12] and FLORES [12] found a positive PAS reaction. The protein content was rather low according to several authors.

Fig. 8. Cytoplasmic area of cell in hyperfunction. The content of the large and dense secretory granules (g) is released into the cytoplasm. The well developed vesicular ergastoplasm (E) and the vesicular and lamellar components of the Golgi apparatus (G) are prominent. Mitochondria (M) possess highly osmiophil membrane. × 15000. *Fig. 9.* Cytoplasmic area in hypofunctional cell, contains remarkably few mitochondria (M) and secretory granules (g). Neither the ergastoplasm nor the Golgi apparatus are seen. × 11000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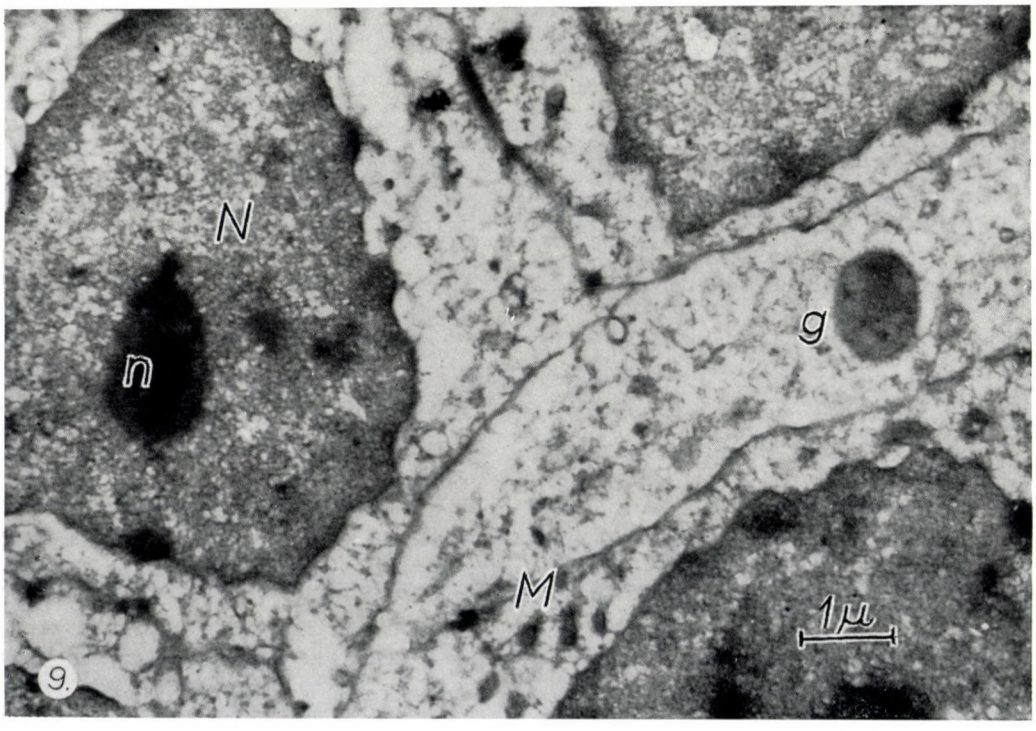
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9.

These findings corroborate our assumption that the Golgi apparatus produces only the markedly osmiophil lipid membrane structures of the secretory vesicles. As it is generally accepted that the development of the secretory product is the result of a complex nuclear-cytoplasmic interaction, our observations concerning the nuclei may be not devoid of interest. The more marked prominence of the nuclear pores described by WATSON [32] and the enhanced structures of the karyoplasm offer morphological evidence for the increased nucleo-cytoplasmic interactions in hyperfunction.

Opposite changes found in parathyroids in hypofunction are also in accordance with the above results. As the transport of secretory granules from cell to cell was never seen but the release into the cytoplasm of the content of the granules was often observed in hyperfunction, it is assumed the product is transported in the form of macromolecules in a way unknown as yet.

The question may arise whether discontinuities in the membranes of the secretory granules are not artifacts due to acrylate explosions. In this case the above explanation of release were invalidated. However, the phenomenon was observed only in hyperfunctional and never in normofunctional glands. On this basis it may be assumed that the discontinuities in the membranes of the granules and the release of product are characteristics of the hyperfunctional glands. Accordingly, these findings are most probably reliable and not due to artifacts.

Summing up the literary evidence and present results the following assumption can be established concerning the mode of secretion. Secretory vesicles are produced by the Golgi ground substance and appear as empty vesicles. They separate from the ground substance and are filled with the secretory product as a result of synthetic processes occurring in the cytoplasm, while the inner density and size increase, until they become secretory granules. As no transfer from cell to cell of secretory granules was observed but release of contents into the cytoplasm takes place in hyperfunction with great intensity — the secrete is presumably transported in a macronuclear form from cell to cell and finally into the blood stream.

It may be further assumed that granules which are formed as discussed above contain but a precursor of the secrete. This precursor undergoes further changes either in the granules or being released becomes the definite product in the cytoplasm of the same or adjacent cells.

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ELEKTRONENMIKROSKOPISCHE UNTERSUCHUNGEN ÜBER DEN SEKRETIONSMECHANISMUS DER EPITHELKÖRPERCHEN VON RANA ESCULENTA

Hyperfunktion der Epithelkörperchen wurde durch, mit EDTA—Na_2-Gabe ausgelöste Hypocalcaemie und Hypofunktion durch mit AT—10 und $CaCl_2$-Verabreichung ausgelöste Hypercalcaemie im Frosch hervorgerufen. Veränderungen der Zellorganellen wurden in elektronenmikroskopischen Untersuchungen verfolgt. Die Ergebnisse zeigten, dass der Sekretionsprozess das Resultat einer komplexen nukleo-zytoplasmatischen Interaktion ist. Den einzelnen Zellorganellen werden folgende Rollen im Sekretionsprozess zugeschrieben. Der Golgi Apparat spielt eine indirekte Rolle durch die Bildung von leeren Vesikeln. Vesikel, welche die Grundsubstanz des Golgi Apparates verlassen, werden im Zytoplasma mit dem spezifischen Sekretionsprodukt oder dessen Präkursor aufgefüllt und dadurch in dunkle und grössere Sekretgranülen umgewandelt.

ЭЛЕКТРОННАЯ МИКРОСКОПИЯ СЕКРЕЦИИ В ОКОЛОЩИТОВИДНОЙ ЖЕЛЕЗЕ ЛЯГУШКИ

Гиперфункция околощитовидной железы лягушки была вызвана экспериментальной гипокальцемией, появляющейся вследствие введения EDTA-Na_2 и гипофункция железы была вызвана гиперкальцемией, наступающей вследствие введения AT—10 и $CaCl_2$. Изменения в отдельных органеллах проследовались в электронном микроскопе. Согласно этим наблюдениям процесс секреции является результатом сложного ядерно-цитоплазматического взаимодействия. Авторы приписывают в процессе секреции отдельным органеллам следующие значения. Аппарат Голджи играет косвенную роль путем образования пустых везикул. Везикулы, мигрирующие из основного вещества аппарата Голджи, наполняются в цитоплазме или специфическим секретом или предшественником его и так они становятся более плотными и увеличенными гранулами секрета.

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SEARCH FOR ANTAGONISTIC ACTINOMYCETAE IN HUNGARIAN SOILS

IV. ANTI-TUBERCULOUS ACTIVITY OF STREPTOMYCES FERMENTATION LIQUIDS

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Synopsis

Fermentation liquids of 361 *Streptomyces* strains isolated from soil samples collected from different geographical regions were tested for antibiotic activity against three virulent strains of *M. tuberculosis* and the non-pathogenic acid-fast strain *M. sp.* M607. Antibiotic activity was demonstrated in 29.8, 25.4 and 17.2 per cent of the fermentation samples against the strains H37Rv, H37Rv (streptomycin-resistant) and H37Rv (isonicotinic acid-hydrazide-resistant), respectively. The M607 strain was inhibited by 5.9 per cent of the samples when tested in liquid medium, and by 12.2 per cent when examined by the solid-testing method. The growth of each of the four strains was completely inhibited by 2.9 per cent.

The above findings show that in the search for antituberculous substances the exclusive use of strain M607 is not satisfactory. Of the fermentation samples inhibiting the strain H37Rv 32 proved to be ineffective against the streptomycin-resistant variant of the same strain. Using the streptomycin-resistant strain in screening tests enables us to select those strains producing streptomycin or streptomycin-like substances. Isolation of the active substances from the most effective fermentation samples is in progress.

Introduction

The appropriate control and eradication of tuberculosis is anu rgent task for Hungary as well as for other countries. For the complex research and therapeutic procedures aiming at the control of tuberculosis the application of chemotherapeutics and antituberculous drugs is of decisive importance.

In the course of chemotherapeutic studies numerous isonicotinic-acid-hydrazide (INH) derivatives have been synthesized, but, unfortunately, none of these had a significant advantage to the basic compound. In the field of antibiotic research, on the other hand, production of nearly 8000 active substances has been reported, but none of these is more effective than streptomycin (STRM) in the therapy of tuberculosis.

The significant results so far obtained and the present knowledge of the biology of *M. tuberculosis* and of the tuberculous organism makes more extensive research and the synthesis of more and more active substances possible and necessary.

For this reason we decided to test the fermentation liquids of antagonistic Actinomycetae obtainable from soils of Hungary and other countries for antituberculous activity.

In general, *M. tuberculosis* is not used as test organism to screen antibiotic strains. Some biological properties of this microorganism, *viz.* the long-lasting cultivation, the questionable homogeneity of the inoculum, and the higher requirement in nutrients make its use unfavourable. Besides, because of the risk of accidental infection special caution is necessary.

Nevertheless, when the object of research is to produce substances active against Koch's bacillus, the use of virulent strains *in vitro* tests is unavoidable.

We carried out experiments in order to extend the applicability of the screening test used in the search for substances active against other microorganisms to the particular case of *M. tuberculosis*. We could not find any report on the use of the same screening test, in spite of thorough survey of the relevant literature.

For the same purpose VÁLYI-NAGY and co-worker [5, 6], WAKSMAN and co-worker [8], and LECHEVALIER and PLEDGER [4] used non-pathogenic strains of *M. tuberculosis*.

As regards chemotherapeutic investigations, the works of YOUMANS, DOUB and YOUMANS [9], LEMBKE and KRÜGER-THIEMER [1], MAYER [3], and of ZSOLNAY [10] should be cited. These authors tested a great number of semiproducts and crystalline compounds against virulent and non-virulent strains.

Materials and methods

The *methods of collecting soil samples*, of the isolation and identification of Actinomycetae, and the techniques of producing fermentation liquid have been published elsewhere [5, 6, 7].

The samples of fermentation were filtered through G—5 bacterium filters and regarding the necessity of transportation and storage, sealed in ampoules under sterile conditions. The contents of the ampoules were used in each case within 24 hours after the sample was taken.

Test organism. M. tuberculosis var. H37 Rv (ATCC); *M. tuberculosis* var. H37 Rv, STRM-resistant (10,000 μg per ml.); *M. tuberculosis* var. H37 Rv, INH-resistant (100 μg per ml.); and *M. tuberculosis* sp. M607 (ATCC).

So-called inoculum suspension was prepared first from each of these strains. For this reason the strains were passaged in modified Šula medium three times [2].

In the case of resistant strains the corresponding inhibiting substances were added to the medium. After three successive passages, *i. e.* after 16 days for the virulent strains and four days for the non-pathogenic cultures, the culture amounting to approximately 150 ml was centrifuged at 3000 r. p. m. The sediment was ground in a "potter" mill. The centrifugation and washing with saline of the sediment were repeated three times, and the final sediment was weighed in a semi-wet state. Then a stock suspension containing 1 mg bacterium per ml prepared. By testing serial dilutions of the suspension the smallest quantities in weight were determined that induced an abundant growth of virulent and non-pathogenic strains within 12—14 and 3 days, respectively. This quantity being 0.05 mg for the H37 Rv strain and 0.005 mg for the non-pathogenic strain was used as inoculum throughout.

As liquid medium modified Šula medium was applied; 4.5 ml were placed in each tube. To this first 0.5 ml fermentation liquid, then the inoculum was added under sterile conditions.

On the solid medium the M607 strain only was used. The agar plate was prepared from pulverized medium enriched with 1 per cent glucose; pH was adjusted to 7.2.

Titration plate like that used in the agar diffusion technique was prepared by inoculating each plate with a suspension containing 0.005 mg M607 in a volume of 0.5 ml. Then pre-

incubation wells of 9 mm diameter were made by a punch, and 0.1 ml of sample of fermentation was placed in each of the wells. After an incubation of 48 hours the inhibition was scored by measuring the diameter of the extinction area.

Sterility test. Glucose broth (1 per cent) was inoculated with each sample immediately after opening the ampoules, under sterile conditions.

pH control. Simultaneously with the sterility test the actual pH of each sample was estimated by using the "Reanal" indicator paper series.

Reading and evaluation of the test. The test was read after 14 and 21 days of incubation for virulent strains and after 48 and 96 hours for the M607 strain.

The cotton stoppers of the tubes were covered with cellophane and this was fixed by a rubber ring to avoid evaporation.

Growth equal to that observed in the control tube was indicated by +++; "complete inhibition" means no bacterial growth in the tubes with sample of fermentation.

Results

The anti-tuberculous activity of the fermentation liquids of 361 *Streptomyces* strains was tested. Of these 16.3 per cent proved to be contaminated by bacteria, most of them by ubiquitous microorganisms, *viz. B. subtilis*, *S. lutea*, and *Staphylococcus albus ;* in four cases Actinomycetes were present.

The pH of most samples ranged from 7.5 to 8.5, in 32 cases between 5.5 and 6.5; in a single sample it was under 4.

The activity of the acidic samples was, in general, low. None of the fermentation liquids with pH 5.5—6.5 were active against all the four test organisms. The percentage distribution of the fermentation liquids as calculated from their inhibiting activity against invidual strains is presented in Table 1.

It is striking that the virulent strains were inhibited by more samples than the non-pathogenic strains. The solid-medium test and the test in liquid medium gave divergent results. Only 18 samples inhibited the growth of the M 607 strain in liquid medium while 37 samples appeared to be inhibitory on the solid medium.

Table 1

Testing of 361 actinomyces filtrates against five strains of M. tuberculosis

	M 607 liquid medium	M 607 plate test	H37 Rv	H37 Rv STRM-resistant	H37 Rv INH-resistant
Active strains					
Number	18	37	90	53	77
Per cent	5.9	12.2	29.8	17.2	25.4

Table 1 also shows that a large number of samples were found to be active against the virulent strains; 90, 77 and 53 liquids inhibited the H37 Rv, the INH-resistant, and the STRM-resistant strains, respectively. The abundant

growth of the virulent strains in the control tubes provided evidence that the inhibition was caused by the active substances present in the fermentation liquids.

Table 2 gives an account of the samples active against more than one of the test organisms. It is seen that the number of samples inhibiting each of the four microorganisms was as small as eight. Thirty-seven liquids inhibited the H37Rv strain, but not the STRM-resistant variant. This finding supports our assumption that the strains producing STRM or STRM-like substances can be selected and consequently, omitted from further studies, by using STRM-resistant test organism. We hope that the use of further test organisms each of which is selectively resistant to one or other well-known antibiotic, such as Neomycin, Viomycin and Kanamycin, will enable us to select the strains producing these drugs. In this manner the number of the strains to be subjected to further analysis can markedly be reduced.

Table 2

Distribution of the filtrates active against two or more test organisms

	Active against all the 4 strains	Active against H37 Rv, not against the STRM-resistant variant	Active against virulent strains, not against M 607	Active against M 607, not against virulent strains	Active against M 607 both in liquid and on solid media
Active strains					
Number	8	37	39	5	18
Per cent	2.9	13.5	14.2	1.8	6.5

The number of fermentation samples that inhibited the virulent strains, but did not inhibit the non-pathogenic M607 strain was 39; the inverse situation was observed in as few as five cases. Consequently, the exclusive use of the M607 strain or any other non-pathogenic *Mycobacterium* does not reflect the in vitro activity against the virulent strains of *M. tuberculosis*.

Eighteen samples elicited antagonistic effect against the M607 strain both in liquid and on solid media. Every strain that inhibited this strain in the former was active when tested on the latter.

Table 3 gives information on the origin of the eight *Streptomyces* strains which proved to be antagonistic against all four test organisms. The quality of the soils and the activity of the strains against other bacteria, *viz. Ps. pyocyanea* and *B. proteus* are also presented. The latter data were obtained from previous studies. As regards the inhibiting activity, no relationship was found between the origin of the strain and the quality of the soil.

The antagonistic activity of four strains was specific to mycobacteria. Strains No. 3668 and No. 3670 had their origin in the same soil; it was shown

by micro-biological tests that these two strains were identical. The antibiotic spectrum of strain No. 2500 is wide; it inhibits *E. coli*, *B. subtilis*, and *Staphylococcus* in addition to the bacteria given in Table 3.

Table 3

Data concerning the strains producing the most active fermentations

No.	Strain No.	Origin	Quality of soil	Activity against			
				M. tubercul.	*Proteus* AH.	*Proteus* X19	*Pyocyanea* CM.
1	2940	Jósvafő (Hung.) . .	silvan	+++	++	—	—
2	2981	Tihany ,, . .	tillage	+++	+++	—	—
3	2983	Tihany ,, . .	tillage	++++	—	—	—
4	2500	Szakállas ,, . .	sodic	+++	+++	+++	+++
5	6038	Szakállas ,, . .	sodic	+++	—	—	—
6	2745	Görbeháza ,, . .	sodic	+++	++	—	—
7	3670	Sochi (USSR)	palm grove	+++	—	—	—
8	3668	Sochi (USSR)	palm grove	+++	—	—	—

Discussion

Clinical practice needs further anti-tuberculous drugs, and the search for such substances needs proper methods. According to our knowledge the method used in the present studies had not been applied for the same purpose. Only crystalline substances, some of them as semi-products had been tested *in vitro* and *in vivo* against Koch's bacillus.

The results obtained by using virulent strains of *M. tuberculosis* as test organisms have brought evidence that a considerable number of Actinomycetae, which are abundant sources of antibiotics, in general, are antagonistic against *M. tuberculosis*.

The practical importance of these experiments is still questionable. YOUMANS [9], LEMBKE [1] and others have shown that the number of substances inhibiting the growth of mycobacteria *in vitro* is high, but most of these substances are ineffective *in vivo*. The present results suggest that the divergencies between *in vitro* and *in vivo* effects of the same substance may be due to the divergent sensitivity of the *in vitro* test organism. The use of virulent strains *in vitro* tests will certainly reduce these divergencies, *i.e.* improve the reliability of the *in vitro* screening tests in search for anti-tuberculous drugs. Further improvement may be expected by introducing resistant strains of *M. tuberculosis* in the screening tests. It is hoped that the tiresome work concerning the isolation of already known antibiotic substances will be avoided in this manner.

The use of Šula's medium was found to be favourable because in this medium the drugs whose effect is suspended or inhibited by protein appear to be inactive.

Most of the chemical and biological characteristics of the active substances of our fermentation liquids are still unknown. Isolation of the active substances from the fermentation liquids listed in Table 3 is in progress.

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DIE FORSCHUNG NACH ANTAGONISTISCHEN AKTINOMYCETEN IN UNGARISCHEN BÖDEN

IV. Untersuchung der antituberkulotischen Wirkung von Streptomycesfermentflüssigkeiten

Aus Bodenproben verschiedener geographischer Herkunft wurden 361 Streptomycesfermentlösungen gezüchtet und ihre antituberkulotische Wirkung gegen *M. tuberculosis* var. H37 Rv, gegen streptomycinresistenten H37Rv-Stamm, gegen den isonikotinsäurehydrazidresistenten H37 Rv-Stamm, sowie gegen den apathogenen, säurefesten Stamm M607 von *Mycobakterium* sp. untersucht. 29,8% der untersuchten Fermentlösungen waren gegenüber dem H37 Rv-Stamm, 25,4% gegen den INH-resistenten H37 Rv-Stamm und 17,2% gegen den streptomycinresistenten H37 Rv Stamm wirksam. Gegen *M. tuberculosis* sp. M607 zeigten in flüssigem Medium 5,9% Fermentflüssigkeiten, bei der Testmethode mit festem Nährboden 12,2% eine hemmende Wirkung. 2,9% der Fermentlösungen hemmten völlig die Vermehrung aller untersuchter Mycobakteriumstämme. Aus den Untersuchungen kann festgestellt werden, dass im Experiment als Wirkstoff gegen Tuberkulose der allgemein gebräuchliche Stamm M607 allein nicht anwendbar ist. 32 auf den H37 Rv-Stamm wirksame Fermentflüssigkeiten erwiesen sich als wirkungslos gegenüber der streptomycinresistenten Variante desselben Stammes. Mit unserer Methode bietet sich bei Anwendung des Streptomycinresistenten Stammes die Möglichkeit zur Ausfiltrierung von Pilzarten, die Streptomycin oder streptomycinartiges Material produzieren. Die Isolierung des Wirkstoffes der sich im Laufe unserer Untersuchungen als höchst wirksam erwiesenen Fermentflüssigkeiten ist im Gange.

ИССЛЕДОВАНИЯ АКТИНОМИЦЕТОВ-АНТАГОНИСТОВ В ПОЧВАХ ВЕНГРИИ

V. Изучение противотуберкулезного действия ферментных жидкостей стрептомицетов

Авторы изучали противотуберкулезное действие 361 ферментных жидкостей стрептомицета на штаммы: *M. tuberculosis* var. H 37 Rv, резистентный к стрептомицину H 37 Rv, резистентный к гидразиду изоникотиновой кислоты H 37 Rv а также на кислотоустойчивый апатогенный штамм *Mycobacterium* sp. M 607. Стрептомицеты были получены из почвенных проб, происходящих с различных географических мест.

29,8% изученных ферментных жидкостей оказало действие на штамм H 37 Rv 25,4% — на резистентный к гидразиду изоникотиновой кислоты штамм H 37 Rv и 17,2% на резистентный к стрептомицину штамм H 37 Rv

На штамм M 607 *M. tuberculosis* sp. в жидкой среде оказало тормозящее действие 5,9% ферментных жидкостей, а при методике на твердой питательной среде — 12,2%.

Размножение всех изученных штаммов *Mycobacterium* прекращалось полностью при действии 2,9% ферментных жидкостей.

Из результатов исследований выясняется, что в исследованиях, направленных на поиск противотуберкулезного действующего вещества, нельзя пользоваться применяющимся вообще одним только штаммом M 607.

Действующие на 32 штаммы H 37 Rv ферментные жидкости оказались недействительными на устойчивый к стрептомицину вариант того же штамма. Применением устойчивого к стрептомицину штамма мы получили возможность для получения сортов грибков, вырабатывающих стрептомицин, или стрептомицинподобное вещество.

В настоящее время ведутся работы изолирования действующего вещества ферментных жидкостей, оказавшихся в наших исследованиях самыми эффективными.

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SEARCH FOR ANTAGONISTIC ACTINOMYCETAE IN HUNGARIAN SOILS

V. EFFECTS OF FERMENTATION LIQUIDS IN VARIOUS IN VITRO TUMOUR TESTS

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Synopsis

The cytotoxic effects of the fermentation liquids of 480 *Streptomyces* isolates obtained from various regions of Hungary were examined in Miyamura's in vitro test; the positive liquids were examined for antibacterial activities. The cytotoxic effects exerted in the Miyamura test and in tissue cultures by the fermentation liquids of 19, morphologically distinct Streptomyces isolates were compared. The peculiar difficulties of anti-tumour antibiotic research are analyzed. Alternation of *in vitro* and *in vivo* tests are suggested in search for active substances of natural origin.

Introduction

In preceding reports of this series [9, 10] an account was given of the incidence of *Streptomyces* strains antagonistic against various pathogenic microorganisms in different soils of Hungary.

Stimulated by works of other investigators [3, 4, 5] we endeavoured to examine for anti-tumour activity the great number of isolates available to us and to obtain some information on the adequacy of the tests applied in the examination of these "natural materials".

MIYAMURA [6] was the first to develop a routine agar-diffusion test for demonstration of *in vitro* cytotoxic and cytolytic effects of synthetic and biological products. Since then the basic principle of this procedure has been applied, with or without modification, by numerous groups of investigators [1]. We demonstrated in a preliminary study [8] that as test organisms for the Miyamura test normal or pathological leucocytes of human origin are as suitable as ascites tumours. The well known tumour-inhibitors also in these conditions exert a strong positive reaction. On the other hand, we found the Miyamura test suitable, first of all, for demonstration of the cytotoxic effect.

The objectives of the present investigations were: (i) to examine by the Miyamura test the tumour-inhibiting effects of the isolates available and (ii) to establish how far the results obtained by the Miyamura test agree with the effects observable in tissue cultures.

Materials and methods

Most of the fermentation liquids examined in the course of the present study had been characterized in earlier reports of this series. The spores preserved in sterile sand were inoculated on a soy-bean medium containing 2 per cent agar. The spores of cultures showing good vegetative growth were suspended in saline by shaking with glass beads.

Two ml of each of these suspensions were used for inoculation of submerged cultures. The composition of the medium applied was published elsewhere [9]. Mycelia from 8-days-old *Streptomyces* cultures were spined down by centrifugation, and the clear supernatants absorbed by filter paper disc of 10 mm in diameter were used in the Miyamura test. Small samples of the fermentation liquids were sterilized by filtration through Jena G5 filters and kept in frozen state befor testing them in tissue cultures.

In order to demonstrate the *in vitro* cytotoxic effects of Streptomyces fermentation liquids, essentially the unmodified Miyamura test was used. Ascites tumour cells were obtained from mice inoculated with the Ehrlich ascites tumour 8 to 10 days before. The final cell concentration of the agar medium was 5.10^6 cells per ml. The filter discs soaked with fermentation liquid were kept on the agar for 10 hours, a period that proved to be sufficient for the cytotoxic substances to diffuse into the medium. The inhibition activity of fermentation liquids acting on the cell dehydrogenases was recorded by reading of diameters of the blue zones appearing around the paper discs after incubating the plates in 37° C thermostate for 120—180 minutes.

Among the fermentation liquids used in Miyamura test only those were considered as cytotoxic which caused an inhibition zonen ot less than 15 mm in diameter. In each of the experiments threefold controls were used: (1) disc absorbed with non-inoculated medium used for the cultivation of streptomyces isolates, (2) a disc soaked in a solution containing 100 μg/ml Degranol* and (3) another disc soaked in a solution containing 10 μg/ml $HgCl_2$.

The medium alone gave never positive reaction. Both the discs of the type 2 and 3 served as positive controls.

The tissue culture tests were carried out as suggested by PÁLYI and GRÉCZI [7]. The fermentation liquids were tested on chicken embryo heart fibroblasts, Crocker's mouse sarcoma cells (S 180) and HeLa cell cultures. The first two sorts of culture were obtained by explanting in sterile test-tubes tissue fragments of 1 to 22 cubic mm on clots produced by mixing chick embryo extract with hen's plasma. Subsequently 1 ml of medium was added. The liquid medium consisted of 5 per cent embryo extract, 10 per cent inactivated horse serum, 85 per cent Hanks' balanced solution, 100 units of penicillin and 100 μg streptomycin per ml.

HeLa cells were cultivated on the flasks' wall. Monolayers were scraped off and cells were suspended in fresh medium. The suspension was distributed in steril tubes, 1 ml in each. The medium consisted of 30 per cent human serum, 70 per cent Hanks' solution, and antibiotics as above.

The cultures were incubated at 37° C in an approximately vertical position. Undiluted or diluted fermentation liquid, 1/10th in volume of the medium, was added to each culture 24 hours after explantation. The tubes were re-incubated for another 24 hours and then examined for cytotoxic changes with low magnification. The degree of cytotoxic effect was estimated on comparison with untreated control cultures. Granulation, rounding and disintegration of cells and the difference in the area of growth were the criteria of cytotoxicity, which was scored as follows.

$\pm$ = initial minimum, slightly increased granulation of the cytoplasm, decreased cell division rate;
$+$ = well-defined lesions of cells; rounded cells at the edges of cultures, the cells are loosened, cytoplasmic vacuolization appears;
$++$ = severe lesion; most of the cells show pathological changes; greatly reduced growth; the contiguity of the cell layer has ceased;
$+++$ = complete destruction of cells which are either rounded or detached from the glass wall;
$-$ = no effects;
$\varnothing$ = not tested.

In the heart fibroblast cultures the rate of growth was measured by means of the ocular micrometer. For examination of the fine cell structure slide cultures were prepared and stained according to Giemsa (heart fibroblast cultures) or with Harris' haematoxylin solution (tumour cells).

* Mannomustine, 1,6-bis/2-chloroethylamino/-1,6-dideoxy-D-mannitol dihydrochloride.

Results

The tumour-inhibiting effects of the fermentation liquids of 480 *Streptomyces* isolates from Hungarian soils were examined by the Miyamura test. Inhibition was shown by 179 liquids (37.2 per cent). Table 1 summarizes the distribution by habitats of tumour-inhibiting *Streptomyces* strains as well as the inhibition of the growth of various test microorganisms due to the same liquids. The data allow to compare the anti-tumour effects with the antibacterial effects of the same materials.

Table 1

Anti-tumour effects of Streptomyces *fermentation liquids demonstrable by the Miyamura test*

Origin of isolate	Number of isolates examined	Fermentation liquids showing anti-tumour activity in the Miyamura test		Coincidence of tumour-inhibition and anti-bacterial effect							
				Liquids inhibiting tumour and Gram-positive bacteria		Liquids inhibiting tumour and Gram-negative bacteria		Liquids inhibiting tumour and Gram-positive and negative bacteria		Liquids inhibiting tumour only	
		No.	%	No.	%*	No.	%*	No.	%*	No.	%*
Békebarlang	74	27	36.4	7	25.9	—	—	—	—	20	74.1
Tokaj-Nyírtelek ..	126	44	34.9	14	31.3	—	—	—	—	30	68.2
Karcag	18	3	16.6	2	66.6	—	—	1	33.3	—	—
Szabolcs-Szatmár .	143	42	29.3	11	26.5	—	—	9	31.7	22	52.3
Aggtelek-Jósvafő .	119	63	52.9	21	33.3	—	—	4	6.5	38	60.2
Total	480	179	37.2	55	30.7	—	—	14	7.8	110	61.4

* As related to the total of tumour-inhibiting fermentation liquids.

The figures in Table 1 suggest that the incidence of *Streptomycetes* qualified as "anti-tumour" effect by the Miyamura test was high in various soils, but only few of the tumour-inhibiting fermentation liquids proved to be antagonistic against bacteria.

Comparative data of 19, morphologically distinct isolates are shown in Table 2. The liquids cytotoxic for one kind of tissue culture were, in general, cytotoxic for the other two as well. In contrast with this, the inhibitions shown by the Miyamura test were inconsistent with those obtained in the tissue cultures in about 50 per cent of the cases.

Table 2

Comparison of anti-tumour effects demonstrable by the Miyamura test and in tissue cultures

Isolate, No.	Dilution of fermentation liquid	Effects on tissue cultures			Miyamura test, inhibition in mm	Correlation between the tissue-culture method and the Miyamura test
		Fibroblast	Crocker	HeLa		
1187	1:10	—*	⌀	⌀	20	—
1005	1:10	- -	—	⌀	—	+
1008	1:10	±	—	⌀	15	—
1140	1:10	—	—	⌀	—	+
1038	1:10	+++	⌀	+++	16	+
1145	1:10	+	⌀	+	—	+?
1000	1:10	+++	⌀	+++	18	+
1066	1:10	++	⌀	+	20	+?
1211	1:10	±	—	—	16	—
1320	1:10	++	+++	++	17	+
1525	1:10	+	+	±	—	—?
1536	1:10	++	+	++	—	—
1786	1:10	++	++	++	—	—
2204	1:10	++	+	++	—	—
2226	1:10	+++	+++	+++	—	—
1310	1:10	+++	+++	+++	18	+
1812	1:10	+++	+++	+++	20	+
1870	1:25	+++	+++	+++	20	+
1913	1:10	+++	+++	+++	15	+

* Explanation in the text.

Discussion

The present studies have shown that a great number of the *Streptomyces* isolates from different regional units of Hungary are cytotoxic in the Miyamura test. In an earlier report of this series [10] the morphological characteristics of the same isolates were analyzed. The morphological variety of the strains excludes the possibility that the cytotoxic isolates represented the same strain.

In accordance with KATO [5] we failed to find any correlation between cytotoxicity and antibacterial effect. We therefore assume that the factors responsible for these two effects are mostly different even in the strains possessing both properties. This assumption is not at variance with the observation that the substance obtained through chemical enrichment of the fermentation liquid of isolate 1320 being positive both in the Miyamura test and in tissue

cultures inhibited the growth of the test microorganisms *B. subtilis* ATCC 6633 as well.

The 19 Streptomyces isolates had not been selected intentionally for the comparative study. For this reason it is remarkable that the 19 isolates frequently exhibited positive effects not only in the Miyamura test, but even in tissue cultures. Though the correlation between the results obtained by the two methodsis incomplete and the number of the strains involved in the study is low, the relationship is not to be overlooked as the strains were different morphologically and the tissue culture studies were performed in three different cell cultures. It can be stated that all the liquids strongly positive in tissue cultures also gave the Miyamura test; on the other hand, in all cases where the two tests gave inconsistent results, the inhibition was only moderate, except for material No. 1187, the strong effect of which in the Miyamura test was presumably nonspecific.

The present study cannot answer the question whether the comparatively often observed tumour-inhibiting capacity of the fermentation liquids was specific or due to the toxic effects of certain metabolites. The fact that only low grade dilutions of liquids were effective favours the latter alternative.

The in vitro tests play a significant role in the search for anti-tumour antibiotics. Of chemotherapeutics of natural origin (antibiotics, phytoncides) only little amounts of the active agent are usually available and the concentration of even a highly active substance may be so low that its activity cannot be demonstrated by means of animal experiments. On the other hand, attempts to concentrate the active substance by physical or chemical means may lead to inactivation of the substance.

Consequently fairly rapid tests requiring minute amounts of active substance are essential in the search for anti-tumour chemotherapeutics. For this reason attention has recently been drawn to the *in vitro* methods although it is well known that *in vivo* and *in vitro* methods often differ. The present results, though based on a small number of experiments suggest that even the two *in vitro* tests most generally used are in disagreement which in our opinion should be ascribed to the inadequacy of the Miyamura test.

When searching for anti-tumour antibiotics rapid screening tests in tissue cultures are preferable, although *in vivo* active substances lacking direct *in vitro* toxicity cannot be recognized by these tests. The observations of DIXON and co-workers [2], *viz.* that the *in vivo* positive filtrates are more toxic in tissue cultures than those found to be ineffective *in vivo*, diminish the significance of this deficiency of the tissue-culture tests.

The fermentation liquids that have been found to be effective *in vitro* should be examined *in vivo*. Animal experiments are to decide whether it is worthwhile to isolate the active substance or not. When biologically assaying single fractions, one should return to the tissue culture, which is the best

suited *in vitro* test. The sample of active substance required by the tissue culture assay is so small and the test is so rapid that complete loss of the active substance must not be feared. Besides, the progress of the isolation procedure should be controlled from time to time animals.

In our opinion there are hardly any available methods more effective in overcoming the innumerable obstacles hindering the anti-tumour antibiotic research than alternation of *in vivo* and *in vitro* tests.

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FORSCHUNG NACH ANTAGONISTISCHEN AKTINOMYCETEN IN UNGARISCHEN BÖDEN

V. Wirkung der Fermentationssäfte auf Tumorkörper in vitro

Die zytotoxische Wirkung des Fermentsaftes von 480 *Streptomyces*-Isolaten aus verschiedenen landschaftlichen Einheiten Ungarns wurde an Miyamura-Methode in vitro untersucht. Verfasser beobachteten ob die positiven Fermentsäfte am Miyamura-Methode über ein antibakteriales Spektrum verfügen. Die zytotoxische Wirkung des Fermentsaftes von 19 morphologisch verschiedenen *Streptomyces*-Isolaten auf Gewebekulturen und auf Miyamura-Methode wurde verglichen. Verfasser analysieren die besonderen Schwierigkeiten der Forschung nach Antibiotika gegen Geschwülste. Es wird die parallele Anwendung der in vitro und in vivo Methoden in der Forschung nach natürlichen Wirkstoffen empfohlen.

ИССЛЕДОВАНИЯ АКТИНОМИЦЕТОВ-АНТАГОНИСТОВ В ПОЧВАХ ВЕНГРИИ

V. Действие ферментативных соков на различные опухолевые тела

Исследовалось цитотоксическое действие ферментативных соков изолятов 480 штаммов стрептомицетов, полученных из различных ландшафтных единиц Венгрии, на Miyamura-метод in vitro. Исследовался вопрос, обладают-ли ферментативные соки,

оказавшиеся на Miyamura-метод положительными, антибактериальным спектром. Сравнивалось цитотоксическое действие ферментативного сока изолятов морфологически различных 19 штаммов стрептомицетов, вызванное на тканевую культуру и на Miyamura—метод. Анализируются особые трудности исследования противоопухолевых антибиотиков. Авторы предлагают параллельное применение in vitro и in vivo методов при исследовании природных действующих веществ.

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SEARCH FOR ANTAGONISTIC ACTINOMYCETAE IN HUNGARIAN SOILS

VI. THE EFFECTS OF BACKGROUND RADIATION ON STREPTOMYCETES

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Synopsis

Twenty *Streptomyces* strains isolated from the radioactive soil (8—10 mμ Curie) of a uranium mine were compared with 20 control strains from inactive soils. Macroscopic, microscopic and electron microscopic morphology of the two groups of strains and their growth on 35 differentiating media were examined. The fermentation liquids were tested for antibiotic activity against *St. aureus* Duncan, *E. coli* 0 111, *Ps. pyocyanea*, *Mycobacterium 607*, *B. subtilis* and the Ehrlich ascites tumour. The radioprotective effects of the liquids were also determined. Between the two groups of isolates only one essential difference was demonstrated, *viz.* out of the *Streptomyces* strains exposed to relatively intensive ionizing radiation for a great number of generations 85 per cent belonged to Pridham's section I while out of the control strains only 26 per cent. Theoretical conclusions were drawn from the results.

Introduction

In earlier reports of this series [9, 10] an account was given of the relationships found between certain chemical properties and the *Actinomyces* flora of the soil. More recently the relations of the physical characteristics of the soil to its *Streptomyces* flora have been investigated in this laboratory. Out of the physical characteristics radioactivity was first chosen for study.

The United Nations' Scientific Committee on the Effects of Atomic Radiation [7] has called attention to the importance of investigations of the living organisms that have been exposed to high background radiation. During the last 15 years intensive efforts were made to clarify the possible somatic and genetic injuries elicited by high dosage of acute short-term radiations, but the somatic, and especially the genetic, consequences of radiations continuing for several generations have remained almost entirely unknown.

MEWISSEN and co-workers [5] studied the biology of higher plants living in regions with high-grade, natural radioactivity. These authors found no difference in radiosensitivity between individuals of *Andropogon filifolius* exposed to radioactive background and those living in inactive areas of Congo when the composition of the two soils was similar.

In our opinion microorganisms are the most suitable to be studied for the biological effects of chronic radiation because their short life cycle permits to observe a long series of generations.

For this reason *Streptomyces* isolates obtained from the radioactive soil of a uranium mine were examined parallel with control organisms originating from inactive soils. We searched for biological characteristics (morphological and biochemical features, antibiotic production) being possibly related to chronic irradiation.

Materials and methods

To detect the soils with appropriate radiation, the G. K. 4 portable apparatus was used. The soil samples were dried, ground and homogenized. The radiation of the homogenized specimens was measured by the 2.8 mg per cm^2 mica-end-window counter. The data thus obtained were compared to the radiation of a uranium standard (U_3O_8) and calculated for 1 g soil sample.

For detailed study 20 isolates from the most active soils were chosen; the activity of these ranged between narrow limits (8.0—10.0 mμ C). Efforts were made to include as many species as possible. Out of the 20 control strains 13 were selected from our international strain collection while 7 were isolated from the inactive soil of the Békebarlang-cave (Jósvafő, Hungary).

The morphology of the strains was assessed according to PRIDHAM's scheme [8]. The shape of sporophores was examined by OKAMI's procedure as modified in this Institute [10]. The electron micrographs were prepared in the Central Laboratory of the University Medical School, Debrecen. The procedures were published elsewhere [4].

The series of media suggested by Waksman served for the determination of biochemical characteristics of the isolates. A few media were modified and some media used by GAUSE were included in the study.

The series of differentiating media was as follows.

1. Czapek agar [13]; 2. glucose-asparagine agar [13]; 3. glycerol agar [13]; 4. tyrosine agar [13]; 5. meat-peptone agar [13]; 6. glucose-peptone agar A [13]; 7. glucose-peptone agar B [13]; 8. meat-peptone gelatin [13]; 9. peptone gelatin [13]; 10. starch agar A [13]; 11. starch agar B [13]; 12. egg-protein agar [13]; 13. potato nutrient agar [13]; 14. potato-glucose agar [13]; 15. starch-nitrate agar [13]; 16. glucose broth [13]; 17. the same without glucose [13]; 18. Czapek solution [13]; 19. starch solution [13]; 20. yeast extract A [13]; 21. yeast extract B [13]; 22. yeast extract agar [13]; 23. Emerson's medium [13]; 24. corn steep [13]; 25. soy-flour medium [13]; 26. synthetic lactate medium [13]; 27. calcium-malate medium [12]; 28. cellulose medium [1]; 29. litmus milk [12]; 30. peptone-gelatin medium for liquefaction test [1]; 31. nitrate medium [1]; 32. potato slice (in 2 ml saline) [1]; 33. carrot slice (in 2 ml saline) [1]; 34. test medium for hydrogen sulfide production [12]; 35. the same without cystine [12].

Four parallel media were inoculated with each of the isolates to observe growth and biochemical responses.

The antibiotic spectra of fermentation liquids were investigated on the test microorganisms used in routine screening tests in this laboratory, *viz. St. aureus* Duncan, *B. subtilis* ATCC 6633, *Ps. pyocyanea* (received from the State Institute of Hygiene, Budapest), *E. coli* 0 111 and *Mycobacterium* 607.

The anti-tumour activity of fermentation liquids was assayed by MIYAMURA's test [6] as modified in this laboratory [11].

The microbiological assay of radioprotective activity was worked out in this Institute [2, 3].

Results

Morphological characterization

The two groups of strains were compared (a) macroscopically, (b) microscopically (sporophores) and (c) electron microscopically (spores).

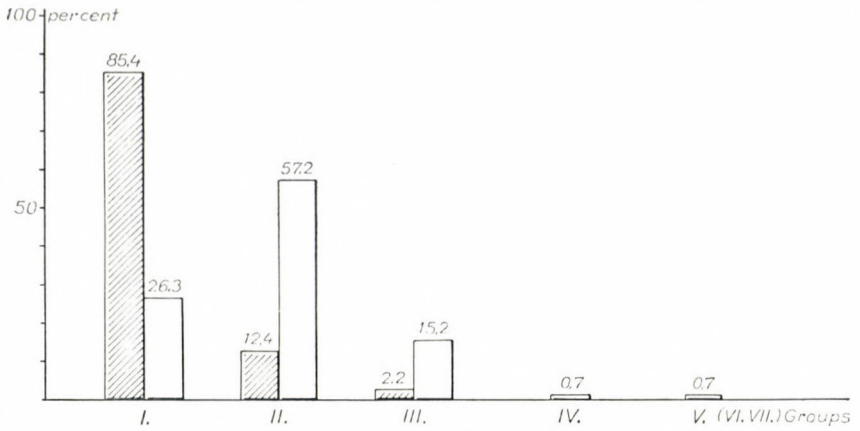
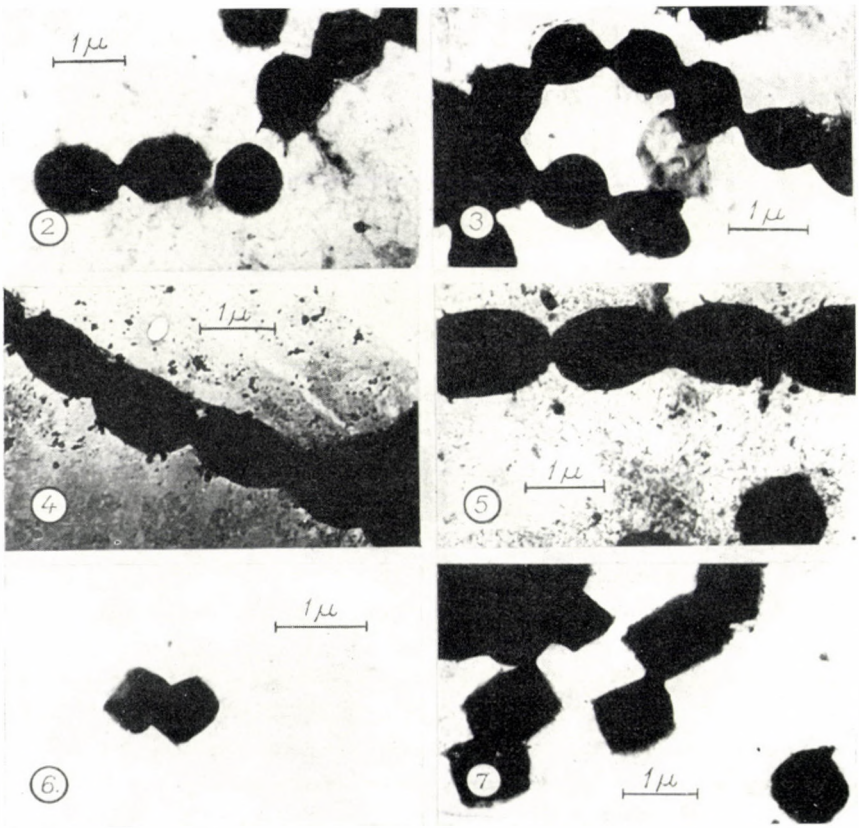


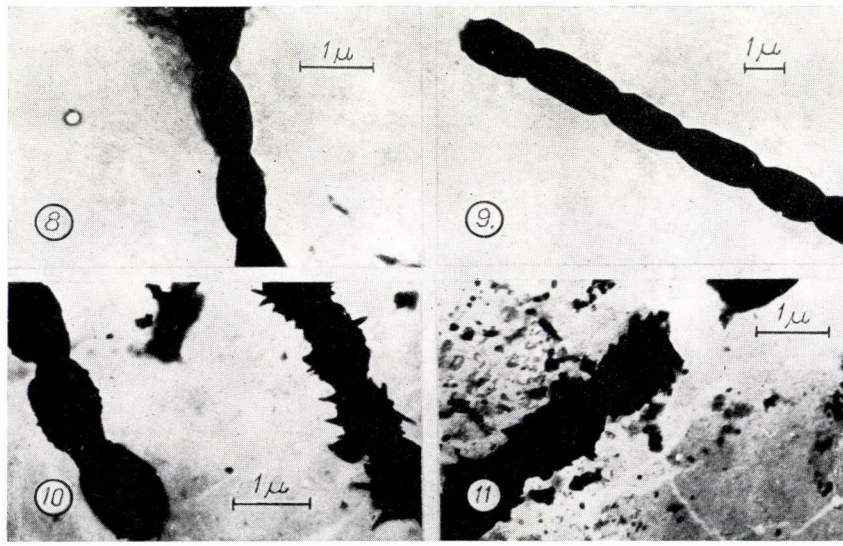
Fig. 1. Per cent distribution of *Streptomyces* isolates according to the PRIDHAM scheme. Shaded columns: 89 strains isolated from radioactive soils. White columns: 145 control strains



Figs. 2 to 7. Electron micrographs of *Streptomyces* spores. There is no difference in the shape of spores between the strains isolated from active soils and the control strains. Round (Figs. 2 and 3), oval (Figs. 4 and 5) and cornered (Figs. 6 and 7) spores are present in both groups

The PRIDHAM scheme was used for classification. As it is well-known, PRIDHAM distinguishes seven sections of the *Streptomyces* subgenus ranging these sections in six series by the colour of air mycelia [8]. Preliminary experiments had shown some difference in the microscopic picture of the sporophores between the two groups. Therefore, in order to obtain statistically appreciable results, the sporophores of 89 "radioactive" and 145 control strains were examined.

It appears from Fig. 1 that, in contrast to the 26 per cent of the control strains, 85 per cent, *i.e.* the great majority, of the strains isolated from radio-



Figs. 8 to *11.* Electron micrographs of *Streptomyces* spores. There is no difference in the spore surface between the strains isolated from active soils and the control strains. Spores both with smooth (Figs. 8 and 9) and spinous surface (Figs. 10 and 11) are present in both groups

active soils belonged to PRIDHAM's section I. The difference is significant, $P<0.1$ per cent. In addition to the quantitative data it should be noted that the "radioactive" strains had sporophores as long and straight as never seen before. Thus, there existed even a qualitative difference in the sporophores.

The electron micrographs (Figs. 2 to 11) show that the spores are variable in size, shape and surface in both groups. Round, oval and cornered spores and spores of smooth or spinous surface were equally found in the two groups, with diameters of spores ranging from 0.6 μ to 1.6 μ.

Biological characterization

To detect further characteristics of isolates, differentiating media were used. Cultivation on the media listed above yielded information on pigment

production and certain biochemical properties, *viz.* utilization of organic and inorganic N and C sources, liquefaction of gelatin, coagulation of milk, hydrolysis of starch and hydrogen sulfide production (Tables 1 and 2).

Detailed analysis of the data revealed no essential difference in the behaviour on these media between the two groups.

Fig. 12 shows antibiotic spectra, anti-tumour and radioprotective activities of the fermentation liquids. Neither the antibiotic spectra nor — in contrast to expectations — the anti-tumour activities and radioprotective effects of the two groups exhibited any significant differences.

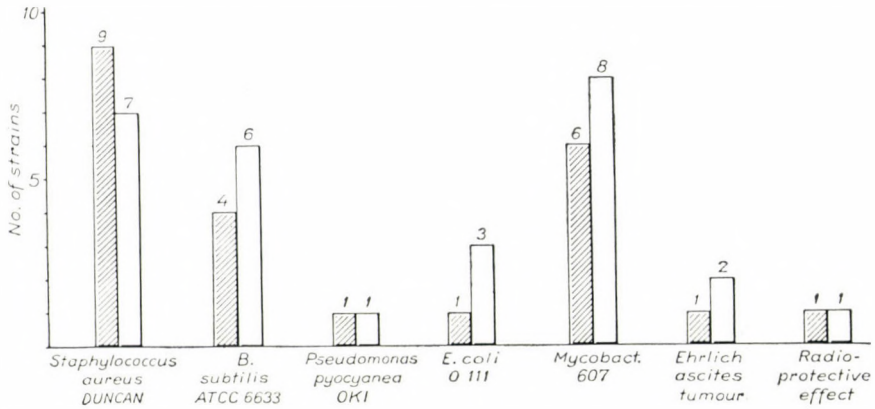


Fig. 12. Antibiotic, anti-tumour and radioprotective activities of *Streptomyces* fermentation liquids. Shaded columns: number of strains obtained from radioactive soils. White columns: number of control strains

Discussion

The present studies revealed but a single substantial difference between the *Streptomyces* strains from radioactive soils and those isolated from inactive backgrounds. The sporophores of the vast majority of the strains which for a series of generations had been exposed to high-level (about 10 mμ Curie) ionizing radiation belong to PRIDHAM's section I, *i.e.* they are straight, do not form bendings or loops or spiral formations. As a contrast only a small proportion of the control strains belong to Pridham's section I on the grounds of their sporophores.

It should be noted that in PRIDHAM's opinion the scheme is based on evolutionary principles, *i.e.* the groups designated with higher numbers are supposed to have attained higher levels of evolution. If so, chronic ionizing radiation would preferably kill cells younger from the evolutionary point of view, *i.e.* the most ancient cells had the best chance to survive. However, this assumption cannot be accepted as a general rule unless confirmed by data to be obtained by finer genetic and biochemical methods on the Streptomyces

Table 1

Cultivation characteristics of Streptomyces

Strain No. / Medium	Czapek agar	Glucose-asp. agar	Glycerol agar	Tyrosine agar	Meat-peptone agar	Glucose-peptone agar A	Glucose-peptone agar B	Meat-peptone gelatin	Peptone gelatin	Starch agar A	Starch agar B	Egg-protein agar	Potato nutrient agar	Potato-glucose agar	Starch-NO_3-agar
103	∅ IV	4 VIII	2 VIII	2 VIII	3	2	4 IV	∅	∅	2	∅	1	4 VIII	∅	2
109	4 IV	3 VIII	1 VIII	2 VIII	3	2	3 IV	3	2	2 VI	3 IV	∅	4	1	2 VI
110	∅	3	2 VIII	2 VIII	3	2	4 IV	2	3	1	2	2	4	2	2 VI
115	∅	2	1	2 VIII	2	1	3 IV	∅	1	2	∅	1	3 VIII	2 IV	4 IV
117	1	3 IV	2 VII	2 VIII	2	3 VIII	4 IV	4	3	4 IV	1	3	3	4 IV	2 IV
120	∅	4 IV	∅	2 IV	2	3	3 IV	2 VI	1	1 VIII	∅	2	3 VI	1	3 VI
125	2	4 VII	2	3	4 IV	2 VI	4 VI	3	2	3	∅	1 VI	3	2	4 V
128	∅	3 IV	∅	2 VIII	3 VIII	3 VIII	3 IV	2	2	3	2	1	2	∅	4 VIII
131	4 IV	3 VIII	1 VIII	1 VIII	2 VIII	3	3 IV	3 IV	2 IV	4 IV	1	3	4 VIII	3	4 IV
136	3 V	4 VIII	2 VIII	2 VIII	4	3	4 IV	1	2	3	∅	1	3 VI	1	3
138	∅	4	3	1	3 IV	3 VI	4 VI	1	3	4	∅	1	2 VI	∅	2
140	2	3	2 VIII	2	2 IV	4	3 IV	1	∅	3 IV	∅	1	2	2 VIII	1
142	2	1 VIII	1	3	1	2	2 VI	∅	∅	2	1	∅	3	1	2
1535/A	3 VI	3 II	1 VIII	3 VIII	4	3 II	4 I	3	2	4 I	3	2	4 VIII	3 IV	4 II
1518	4 VI	4 II	2 VIII	2 VIII	2	2 II	4 I	4	3	4	3	4	3 VIII	1 IV	3 I
1919	4	4	2	2 VIII	4 IV	3 IV	4 VIII	3	2	4	1	3	4	2	4 VII
1929	2	4 II	2	3 VIII	2	2	4 II	2	2	4	1	3	3 VIII	2	3 II
1931	4 IV	4 IV	2	3 VIII	4	3	4	2	2	4	∅	2	3	∅	4 IV
1944	2	4	1	2 VIII	4	3	4 II	2	2	4	∅	1	2 VIII	3	2 II
1995	3 V	4 IV	2	3	4	3	4 IV	1	1	4	∅	1	2	2 IV	4 VII

Key to the signs used

1: minimum growth
4: maximum growth
*: hydrogen sulfide production

I: blue pigment
II: red pigment
III: green pigment

isolates obtained from inactive soils

Glucose broth	Idem without glucose	Czapek solution	Starch solution	Yeast extract A	Yeast extract B	Yeast extract agar	Emerson's medium	Corn steep	Soy-flour medium	Synthetic lactate medium	Ca malate medium	Cellulose medium	Litmus milk	Peptone-gelat. liquef.	Nitrate medium	Potato slice	Carrot slice	Medium for H_2S production	Idem without cystine
3	2	1	3 II	2	2	2	2	1	3	2	1	∅	—	∅	2 IV	3	—	3	2 VIII
2	2	1	2	2	1	1 IV	2	1	1	∅	3	∅	1	∅	3	3	—	3	3
2	3	2	1	3	2	3	1	2	2	∅	3	∅	1	∅	1 IV	3	—	3	2
2	2	1	2	2	2	3 VI	2	1	3	∅	3	∅	1	∅	1	3	—	2	3 VIII
2	2	2	2	3	2	2	1	1	3 VI	∅	4	1	—	2	3	2	1	3	3
3	2	1	2 IV	3	3	2	2	1	2	1	4	∅	1	∅	1	2	—	∅	∅
2 VI	2 VI	2	2	3	4 VI	1	3	1	3	1	4	∅	—	∅	3 III	2	—	2* VI	2*
4	4	1	2 IV	2	1 VI	1 V	2 IV	1	1	1	2	∅	1	3	1	2	—	3	2
2	2	3	2	3	4	2	2 VI	1	4	∅	3	1	—	∅	2	4	2	2	3
2	3	2	3	2	3	4	1	2	1	∅	3	∅	1	∅	2	3	2	3	3
2	1	1	4 II	2	1	2	2	1	2	∅	2	∅	—	∅	3	2	—	2* VI	3*
2	1	1	2 IV	3	2	2	2	2	1	1	3	∅	—	∅	2	2	—	2* VI	3*
2	2	2	1 II	4	1	1	1	1	2	∅	2	∅	—	∅	1	3	2	1	3
2 II	4 II	1 II	3	3	3	4 II	3	1	4	2	4	∅	1	3	2	4	2	2* VI	3
2 II	2 II	1 II	2	4	4 VII	4	1 II	4 II	4	3	4	1	1	2	2 II	4	—	2 VIII	2
3	4	2	3	3	4	3	4	2	2	1	3	∅	1	∅	2	4	—	4	3
2 II	4 II	1	2	2	4	3	2 II	4	2	∅	2	∅	—	∅	2 II	3	3	2	2
4	4	1 II	2 II	2	4	2	2	4	3	1	3	∅	1	2	2	2	3	3	2
3 II	3 II	2	1	1	4	4	3	4	3	1	3	∅	1	∅	3 II	2	—	2	2
3 II	4 V	3 II	3 V	3	4 VI	2	3	1	4	∅	2	∅	1	∅	2 II	4	—	3*	3

IV: yellow pigment
V: grey pigment
VI: brown pigment
VII: purple pigment
VIII: no sporulation

Table 2

Cultivation characteristics of Streptomyces

Strain No. / Medium	Czapek agar	Glucose-asp. agar	Glycerol agar	Tyrosine agar	Meat-peptone agar	Glucose-peptone agar A	Glucose-peptone agar B	Meat-peptone gelatin	Peptone gelatin	Starch agar A	Starch agar B	Egg-protein agar	Potato nutrient agar	Potato-glucose agar	Starch-NO_3 agar	Glucose broth
2355	4	3	3	1	1	2	3	4	2	3	1	2	4	2	4	3
2359	4	3	4	3	4	3	1	3	2	4 IV	2 III	4 IV	3	3 V	4 VI	2
2360	1	3	1	∅	∅	4 VI	3 III	2 VI	3	3	∅	3	4 VI	∅	3 VI	3
2390	4	3	4 VI	1	∅	4 VI	3 VI	4	3	4 VI	1	2 VI	4	∅	3 VI	3
2396	4	1	2	∅	∅	1	1	4 V	2	3 V	1	3	4	∅	1	2
2400	3	3	3 VI	1	1	4 VI	4	4 VI	1	4	∅	4 III	4 VI	4	4 III	3
2408	3	2	2	∅	∅	3	3 VIII	1 VIII	∅	3 VII	∅	1	2	3	1 VII	3
2433	3	2	4	1	∅	4 VI	4 VI	4 VI	2	4 VI	∅	3 V	4 VI	3	3 VI	3 VI
2259	4	3	4	3	1	3	3	4	4	4 V	2	3 V	4 IV	3	4 IV	3
2351	3	3	3 II	1	1	2	3	4	2 V	3	1	3	4 V	2	4 IV	3
2353	3	3	4 II	1	1	3	2	4	3	4 V	1	1	4 V	2	4 IV	3
2381	2	2	2	∅	∅	2 VI	∅	3	2	2	1	3	2	∅	1	2
2407	3	3	4 VI	3	∅	4 VI	4	4 VI	∅	4	2	4 III	4 VI	4 III	3	3
2432	4 III	3	3 VI	∅	1	3	3	4 VI	3	4 III	2	2	4 VI	4 III	2	3
2361	4	1	3	∅	∅	1	∅	4	2	3	∅	1	4	1	1	1
2362	4	1	3	∅	∅	1	∅	1	2	3	∅	2	4	∅	1	1
2364	4	1	3	∅	∅	1	∅	4	2	3	∅	2	4	∅	1	1
2371	4	1	3	∅	∅	1	∅	4	2	3	∅	1	4	∅	4	1
2379	3 III	3	3	∅	∅	3	2	4	3	3	1	1	3 VI	∅	2	3
2382	3 I	1	3	∅	∅	1	1	4 V	2	3 V	1	2 V	4 V	∅	2	1

* See footnote to Table 1.

*isotates obtained from active soils**

Idem without glucose	Czapek solution	Starch solution	Yeast extract A	Yeast extract B	Yeast extract agar	Emerson's medium	Corn steep	Soy-flour medium	Synthetic lactate medium	Ca malate medium	Cellulose medium	Litmus milk	Peptone-gelat. liquef.	Nitrate medium	Potato slice	Carrot slice	Medium for H_2S production	Idem without cystine
2	3	3	3	3	2	3	3	3	1	3	∅	1	∅	2	2	—	2 VIII	2 VIII
2	2	3	3	3	4 VI	3	3	2	∅	3	∅	1	∅	4	4	—	3	3
2	3	3	3	3	2 VII	3	3	3	1	3	∅	—	∅	2	4	—	3 VIII	3 VIII
1	4	3	3	3	4 VI	4	2	3	1	3	∅	—	∅	3	4	—	3 VIII	3 VIII
2	4	4	3	1	3	1	2	2	1	3	∅	1	3	2	4	3	4 VIII	3
3	3	3	3	3	4 VI	3 VI	2	3	1	3	∅	1	∅	2	4	1	4 VIII	3
3	2	3	2 VI	1	1	2	2	3	∅	4	∅	1	∅	2	4	2	3	3 VIII
3	3	3	2 VI	2	3	3 V	2	3 VI	1	3	∅	1	∅	4	4	1	4* VIII	4* VIII
1	3	3	3	3	3 V	3 VI	3	3	2	3	∅	1	∅	2	4	—	4*	3
2	2	3	3	3	2 V	2 V	3	3	1	3	∅	1	∅	3	4 III	—	3	3 VIII
2	2	3	3	3	2 V	2 V	3	2	1	3	∅	1	3	3	4	—	3 VIII	3 VIII
1	2	3	2	2	1	1	1	2	∅	4	1	1	∅	2	4	1	3	3
3	3	3	3	3	4 VI	3	2	3	2	4	∅	1	∅	2	4	3	4	4 VIII
3	3	3	4 VII	3	3 III	4 III	2	1	1	3	∅	1	3	2	4	3	3 VIII	3 VIII
1	3	3	2	1	3	2	1	1	∅	3	∅	—	∅	2	3	1	3 VIII	3 VIII
1	3	3	2	1	3	1	1	1	∅	3	∅	—	∅	3	3	4	3 VIII	3 VIII
1	3	3	2	1	3	3	1	1	∅	3	∅	—	∅	3	4	3	3 VIII	3 VIII
1	3	3	2	1	3	2	1	1	∅	3	∅	1	∅	1	2	2	3 VIII	3 VIII
1	2	3	3	2	3	3	3	2	1	3	∅	—	∅	2	2	—	3 VIII	3 VIII
1	4	4	3	1	3 V	3 V	2	2	1	3	∅	—	∅	2	2	1	2 VIII	3 VIII

floras of the radioactive soils of numerous geographical areas remote from each other.

We had hoped to find in the radioactive soil more strains producing radioprotective substances. It seems, however, that the higher radioprotective activity of certain Streptomyces fermentation liquids should be attributed to the higher metabolic activities of the strains involved rather than to an adaptation mechanism.

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FORSCHUNG NACH ANTAGONISTISCHEN ACTINOMYCETEN IN UNGARISCHEN BÖDEN

VI. Die Wirkung der intensiveren Hintergrundaktivität auf Streptomyces

Zwanzig aus dem radioaktiven Boden eines Uranbergwerkes (8—10 mμ Curie) isolierte *Streptomyces*-Stämme wurden mit ebenso vielen aus inaktivem Boden erhaltenen Stämmen verglichen. Die makroskopische und elektronenmikroskopische Morphologie der Stämme wurde untersucht. Die Beobachtung des Verhaltens der beiden Gruppen erfolgte an 35 verschiedenen elektiven Nährböden. Das antibiotische Spektrum der Fermentsäfte der untersuchten Stämme gegenüber *Staphylococcus aureus*, *E. coli* 0111, *Ps. pyocyaneus*, *B. subtilis* M. 607 wurde aufgenommen und durch die Untersuchung ihrer Wirkung auf Ehrlichschen Aszites-Tumor sowie der Strahlenschutzwirkung ergänzt. Auf dieser Grundlage gelang es

zwischen den beiden Gruppen einen wesentlichen Unterschied aufzuzeigen. Von den viele Generationen hindurch einer intensiveren ionisierenden Strahlung unterworfenen *Streptomyces*-Stämmen gehörten 85% in die erste Pridhamsche Gruppe, gegenüber 26% der Kontrollgruppe. Aus den Ergebnissen wurden theoretischen Folgerungen abgeleitet.

ИССЛЕДОВАНИЕ АКТИНОМИЦЕТОВ-АНТАГОНИСТОВ В ПОЧВАХ ВЕНГРИИ

VI. Действие более интенсивной радиоактивности фона на штаммы стрептомицетов

Авторы сравнивали 20 штаммов стрептомицетов, выращенных из радиоактивной почвы уранового рудника (8—10 mμ Curie) с контрольными штаммами, полученными из инактивной почвы. Изучалась макроскопическая и электронномикроскопическая морфология исследуемых штаммов. Авторы наблюдали над поведением двух групп на 35 различных избирательных питательных средах. Они снимали антибиотический спектр ферментативных соков исследуемых штаммов против ***Staphylococcus aureus*** Duncan, *E, coli* 0 111, *Ps. pyocyaneus*, *B. subtilis M.* 607, и исследовали их действие на асцитическую опухоль Эрлиха и защитное действие против излучения. На основании проведенных экспериментов удалось выявить значительное различие между двумя группами; 85% штаммов ***Streptomyces,*** подверженных в течение многих поколений действию ионизирующего излучения более высокой интенсивности, относилось к первой группе Придхама, в противоположность контрольным штаммам, из которых только 26% относилось к этой группе. Из полученных результатов авторы сделали теоретические заключения.

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COMPARATIVE DATA AND ENZYME KINETIC CALCULATIONS ON CHANGES CAUSED BY TEMPERATURE IN THE DURATION OF GASTRIC DIGESTION OF SOME PREDATORY FISHES

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Synopsis

Conducting gastric digestion experiments the authors examined the length of time required for gastric digestion by pike perch *(Lucioperca lucioperca* L.), perch *(Perca fluviatilis* L.), largemouth bass *(Micropterus salmoides* LACÉPÈDE) and sheatfish *(Silurus glanis* L.) at 5, 10, 15, 20 and 25° C. With a view to comparison, the data pertaining to all four species were expressed in per cent of the digestion period registered at 5° C. As to the effect of temperature on the duration of gastric digestion considerable differences were found in the examined species. It was pointed out that the curve of gastric digestion plotted against temperature may be characterized by a quadratic equation, and that with increasing temperature the period of gastric digestion diminishes according to geometrical progression. The duration of gastric digestion decreased from the values established at 5° C to 11 per cent in pike perch, to 18 per cent in perch, to 17 per cent in largemouth bass and to 10 per cent in sheat fish when a temperature of 25° C was applied. The data were also evaluated from the aspect of ferment kinetics. The graphic representation of experimental records by the ARRHENIUS equation yielded straight lines from which the activation energy could be calculated.

Introduction

In the course of examining the natural food of pike perches living in Lake Balaton and weighing 300 to 500 g conspicuously many starving individuals with empty stomach were found [10]. Searching into the cause and degree of starvation it had to be clarified what time the food-filled stomach of pike perch needs to become empty in different seasons and, consequently, what is the frequency of food uptake. With the field methods of ecology these questions could not be solved, therefore gastric digestion experiments were established at different temperatures.

Applying the X-ray method [2] elaborated to observe the emptying of pike perch stomach and conducting experiments at 5, 10, 15, 20 and 25° C the changes caused by temperature in the duration of gastric digestion were assessed. The data thus obtained express the time necessary for softening (pulpifying) the food fishes of known size and reveal what time the decomposition by pepsin takes at different temperatures in the hydrochloric medium

of pike perch stomach. Illustrating graphically the mean values, the temperature curve of gastric digestion period in pike perch was obtained [3, 4]. From this curve and from the proportion of the results of temperature experiments carried on with pike perch conclusions may already be drawn as to the frequency of food uptake and its seasonal changes as influenced by temperature.

Pike perch experiments confirmed the validity of the radiographic method, therefore, considering also the possibility of interspecific comparison, similar investigations were started with perch, largemouth bass and sheatfish [5, 6].

Temperature dependency of gastric digestion time

The temperature curve constructed from experimental data pertaining to the duration of gastric digestion in the tested fish proved unsuitable for direct comparison. This was due the fact that from the four species only specimens of different size were at disposal, the ratio between the weight of the experimental fishes and the food fish (*Alburnus alburnus* L. and *Acerina cernua* L.) was different for the four species. Therefore it had only to be established, to what degree temperature changes affect the duration of softening process in the stomach of the four fish species.

Having finished the investigations, for the sake of comparison a common starting point had to be chosen. Because within the same species always entirely identical experimental conditions were provided for, it seemed right to compare the data obtained for the four fish species by expressing them in per cent.

Computation was based in case of all four species on the mean of gastric evacuation time registered at 5° C while the records obtained at the other four temperatures were expressed in per cent of the 5° C values. The comparison of percentual ratios and the curves constructed by the aid of these numbers denote clearly the differences existing among the four fish species.

The comparable percentual data are presented in Table 1, showing the basic values obtained at 5° C also in hours (between brackets).

A graph was constructed from the data of Table 1 (Fig. 1). For all four fish species the curve was plotted in a system of coordinates showing the percentual values of gastric digestion period as the function of temperature. On the ordinate the percentual data of gastric digestion period registered at 5° C, on the abscissa temperatures were plotted. Curves originate naturally at the same point (5° C = 100). Their further course demonstrates the effect exerted by temperature changes on the duration of gastric digestion and reveals that in this respect great differences exist among the four fish species.

A comparison of the curves led to following statements.

(1) It attracts attention immediately that a rise of temperature increases the intensity of gastric digestion mainly in sheat fish, whereas in perch the smallest "acceleration" may be observed.

Table 1

Percentual changes of gastric digestion period on the basis of the values obtained at 5° C

C°	Pike perch	Largemouth bass	Sheat fish	Perch
	per cent (hours)			
5	100 (257h)	100 (110h)	100 (206h)	100 (115h)
10	61	46	42	55
15	32	34	24	43
20	18	22	14	24
25	11	17	10	18

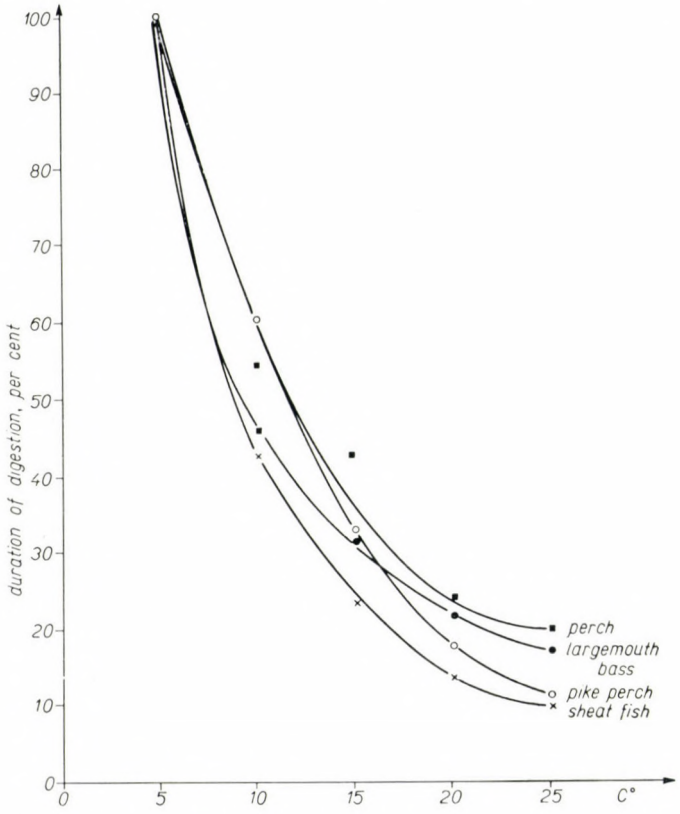

Fig. 1. Changes in percentual data of gastric digestion period, taking the values registered at 5° C as a 100 per cent basis (pike perch = *Lucioperca lucioperca* L; perch = *Perca fluviatilis* L.; largemouth black bass = *Micropterus salmoides* LACÉPÈDE; sheat fish = *Silurus glanis* L.)

(2) In pike perch, perch and sheat fish the decrease of gastric digestion period shows — towards 25° C — an equal rate. In largemouth bass the increase of intensity is very vigorous at lower temperatures, and disminishes considerably between 15 and 25° C.

(3) Gastric digestion in sheat fish and largemouth bass accelerates specially between 5 and 10° C, whereas in pike perch and perch the acceleration is hardly greater in this low temperature range than between 10 and 15° C.

(4) The stomach of sheat fish and pike perch digests approximately ten times faster at 25° C than at 5° C. With largemouthed bass and perch the same comparison shows only a five to sixfold difference.

(5) Due to rise of temperature the duration of gastric digestion diminishes according to geometric progression, with major or minor differences per species.

Enzyme kinetic calculations

The statement presented under paragraph 5 of the previous chapter rendered the supposition probable that the connection observed between temperature and gastric digestion of the tested predatory fishes reflects a simple interdependence of ferments and temperature. This was confirmed by having obtained quadratic empirical curves [3, 4, 5, 6], and by the fact that in the stomach of predatory fishes a simple decomposition by pepsin and hydrochloric acid takes place.

According to JOHNSON, EYRING and POLISSAR [1] and SIZER [7] several complicated biological processes are similar to ferment kinetic relations observed in vitro. These authors pointed out that many biological processes differing from each other entirely, are in complete conformity with the following equation well known in chemistry and applied first by ARRHENIUS:

$$A = (\ln k_2 - \ln k_1) \cdot R \cdot \frac{T_1 \cdot T_2}{T_2 - T_1}.$$

This indicates that the connection existing between the natural logarithms of a kinetic velocity constant and the reciprocal values of absolute temperature is represented by a straight line, the directional angle of the latter being the thermal coefficient from which the activation energy may be calculated.

The durations of gastric digestion registered at different temperatures were considered as separate "reaction-kinetic velocities" which may be expressed by using reciprocal values.

Graphing the natural logarithms of the reciprocal values of digestion time in the sense of the Arrhenius equation a surprisingly close linear regression was obtained (Fig. 2), and also statistically checked for four and five points respectively.

The latter procedure assessed especially good linearity for the 10 to 25° C temperature range. Accordingly it may be stated that *the gastric digestion period of predacious fishes measured at different temperatures changes in compliance with the Arrhenius equation.* It is interesting to note that this was confirmed also by the values pertaining to the gastric digestion of *Cobitis fossilis* established by VONK [8, 9].

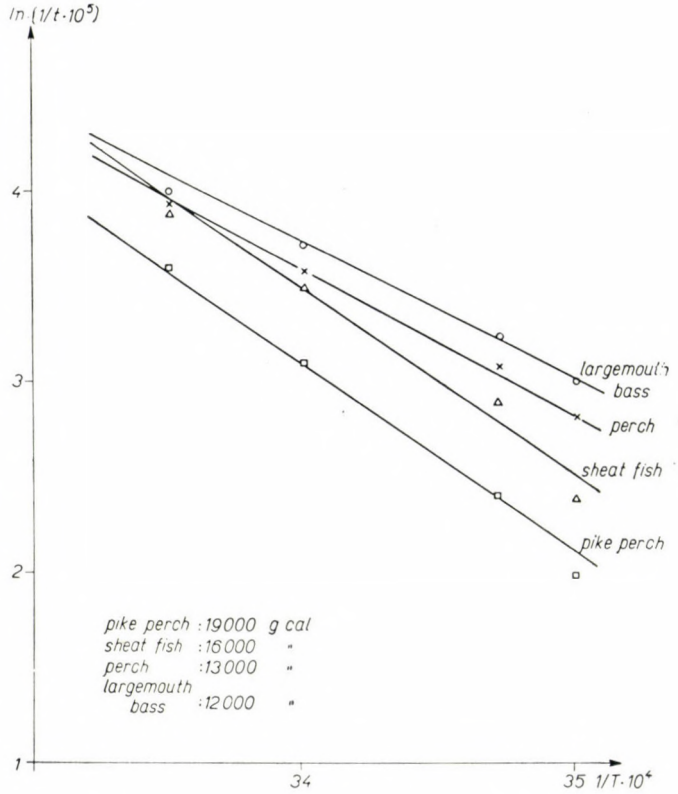

Fig. 2. Values of activation energy exerted by the gastric digestion of different predatory fish species (pike perch = *Lucioperca lucioperca* L.; perch = *Perca fluviatilis* L.; largemouth black bass = *Micropterus salmoides* LACÉPÈDE; sheat fish = *Silurus glanis* L.)

According to calculations the tested fish species perform gastric digestion with following — rounded off — amounts of activation energy (precise results of calculation are given within brackets).

Cobitis fossilis	20.000 (20 257.01)	gcal
Lucioperca lucioperca	19.000 (18 970.33)	,,
Silurus glanis	16.000 (16 001.06)	,,
Perca fluviatilis	13.000 (12 701.87)	,,
Micropterus salmoides	12.000 (11 547.16)	,,

SIZER [7] proved by in vitro experiments that pepsin decomposes casein with 18,000 gcal. Naturally, the authors do not ascribe higher importance to their own figures representing as absolute values. However, in the above mentioned interesting coincidence of data the secondary physiological consequences and comparison of activation energy values must be emphasized

(The findings of the authors are not influenced by the validity or wrongness of CROZIER's theory either, because the trend manifesting itself in a relatively broad heat interval is obviously linear.)

Grouping and sequence of data is all the more noteworthy. Among the four species largemouth black bass is the sprightliest, most rapturous fish showing the lowest, *i.e.* most favourable degree of activation energy. This may perhaps partly be explained by the fact that the stomach of largemouth black bass exerts a vigorous crushing movement too and has many pylori increasing its surface. Perch and pike perch do not agitate ingested small fishes at all.

Conclusion

Summarizing the results, the general ecological regularity became evident that despite of an eventual greatest food abundance for predacious fishes in winter, ferment kinetics relentlessly interferes with the duration of gastric digestion. From this fact pisciculture has to draw its practical conclusions.

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VERGLEICHENDE ANGABEN UND FERMENTKINETISCHE BERECHNUNGEN ÜBER DIE TEMPERATURBEDINGTE DAUER UND ÄNDERUNGEN DER MAGENVERDAUUNG EINIGER RAUBFISCHARTEN

Verfasser studierten mit bei Temperaturen von 5, 10, 15, 20 und 25° C durchgeführten Magenverdauungsversuchen die Dauer der Magenverdauung beim Zander *(Lucioperca lucioperca* L.), Barsch *(Perca fluviatilis* L.), Forellenbarsch *(Micropterus salmoides* LACÉPÈDE) und Wels *(Silurus glanis* L.). Die Ergebnisse wurden vergleichshalber bei allen vier Arten in Prozenten der bei 5° C erhaltenen Angaben über die Verdauungszeit ausgedrückt. In der durch die Temperatur auf die Länge der Verdauungsperiode ausgeübten Wirkung zeigten sich bei den untersuchten Fischarten beträchtliche Unterschiede. Es wurde festgestellt, dass die Kurve der Magenverdauung als Funktion der Temperatur mit einer quadratischen Gleichung charakterisiert werden kann und die von ihr beanspruchte Zeit mit steigender Temperatur einer geometrischen Progression folgend abnimmt. Wenn eine Temperatur von 25° C angewandt wurde, verringerte sich die Magenverdauungszeit beim Zander auf 11%, beim Barsch auf 18%, beim Forellenbarsch auf 17% und beim Wels auf 10% der bei 5° C ermittelten Werte. Die Ergebnisse wurden auch vom fermentkinetischen Blickpunkt gewertet. Die Versuchsangaben lieferten — nach der Gleichung von Arrhenius dargestellt — Geraden, aus denen sich die Aktivisierungsenergie berechnen lässt.

СРАВНИТЕЛЬНЫЕ ДАННЫЕ И ФЕРМЕНТО-КИНЕТИЧЕСКИЕ ИСЧИСЛЕНИЯ ИЗМЕНЕНИЙ ВРЕМЕНИ ЖЕЛУДОЧНОГО ПИЩЕВАРЕНИЯ, ВЫЗВАННЫХ ТЕМПЕРАТУРОЙ У НЕКОТОРЫХ ВИДОВ ХИЩНЫХ РЫБ

Авторы в опытах по желудочному пищеварению исследовали у судака (*Lucioperca lucioperca* L.) окуня (*Perca fluviatilis* L.) большеротного черного окуня (*Micropterus salmoides* LACÉPÈDE) и у сома (*Silurus glanis* L.) в ремя желудочного пищеварения при температуре 5, 10, 15, 20 и 25° C. В интересах сопоставления, полученные данные в отношении всех четырех видов рыб выражаются в процентах времени переваривания, полученного при 5° C. В действии температуры на время переваривания у исследованных видов животных авторы наблюдали значительные различия. Установлено, что кривую температуры-желудочного пищеварения можно выразить уравнением второй степени и что при повышении температуры время желудочного пищеварения снижается по геометрической прогрессии. Полученная при 25° C величина составляет у судака 11%, у окуня 18%, у большеротного черного окуня 17%, а у сома 10% времени желудочного пищеварения, полученного при 5° C. Авторы подвергают полученные данные также ферменто-кинетическому анализу. Если изобразить экспериментальные данные при помощи уравнения Аррениуса, получаются прямые, по которым можно вычислить энергию активизации.

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EPENDYMOSECRETION (EPENDYMAL NEUROSECRETION)

V. THE CORRELATION BETWEEN GLIAL CELLS CONTAINING GOMORI-POSITIVE SUBSTANCE AND EPENDYMOSECRETION IN DIFFERENT VERTEBRATES

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Synopsis

The glial cells containing Gomori-positive substance of the central nervous system of amphibia, birds and mammals have been studied. The cells occur in every species examined by the authors. The Gomori-positive cells may be divided into two distinct groups: (1) Periventricular glial cells, located under the ependyma, possessing few processes, and having polymorphous nuclei low in chromatin. (2) Perivasal type. These are cells possessing oval nucleus, rich in chromatin, and more processes, occurring equally in different parts of the brain around the capillaries and veins. The periventricular type of glial cells is correlated with the ependymosecretory system as regards both morphology and genesis. In the neurosecretory cell groups the perivasal type of cells can be found.

Introduction

Ependymosecretion or ependymal neurosecretion is understood to mean that phenomenon when the ependyma produces a Gomori-positive substance [13]. The most classic example of this is the secretion of the epithalamic subcommissural organ [12]. A similar phenomenon is detectable in the hypothalamic ependyma, too [13]. In this area the process is significant because the ependymosecretory material reaches the hypophysial portal blood vessels. Thus, the substance may be involved in the hypothalamic control of endocrine activity [14], which is at present in the focus of interest in neuroendocrinology [1, 11].

Thus far, in the hypothalamus the neurosecretory substance as the best known Gomori-positive hypothalamic substance, has been the object of most extensive study. However, beside neurosecretory and ependymosecretory material there exists also a third, morphologically different Gomori-positive substance: the Gomori-positive granules accumulating in certain glial cells. Smith was the first in 1951 to find glial cells — containing Gomori-positive substance — in the hypothalamus of vertebrates [10]. Diepen [4] mentions that similar cells could be found subependymally in adult mice. He thinks, just like Noda, as well as Löfgren later [9, 7] that the substance in the glial

cells is ingested neurosecretory material. In contrast to this, HILD [5] claims that such cells occur not only in the hypothalamus, but in other parts of the brain as well; and the granules of the cells can be stained with Gomori's method even after treatment with alcohol-chloroform, or acetone, whereas the neurosecretory substance does not stain after such treatment. From all these he concludes that the granules of the glial cells are not identical with the neurosecretory material. BARRY [2], too, thinks that the granules of the glial cells are different from neurosecretion. The problem has been studied in more detail by WISLOCKI and LEDUC [17, 18]. As early as 1952, the authors divided the Gomori-positive glial cells in the rat in two groups: the true glial cells, found directly under the ependyma, around the third ventricle and the Sylvian aqueduct, and the macrophage-like cells, that occur throughout the brain perivascularly and in the meninges. They did not mention any correlation between the periventricular glial cells and the neurosecretory nerve cells.

In our earlier investigations we, too, found similar cells under the ependymal cells [13], in such areas in which the ependyma produces Gomori-positive material. Thus, it has been suggested that a correlation may exist between the Gomori-positive glial cells and ependymosecretion.

To investigate the problems that have emerged, in the present work we have studied in different vertebrates the glial cells containing Gomori-positive substance. We have been seeking answers to the following questions: (*a*) Do the glial cells mentioned occur in every species examined by us? (*b*) What is the more detailed morphology of the cells like? (*c*) Is there a morphological correlation between these cells and ependymosecretion or neurosecretion? A short abstract has been published elsewhere [15].

Materials and methods

The following species have been involved in the investigations: *Triturus cristatus*, *Triturus vulgaris*, *Pleurodeles waltlii*, *Amblystoma mexicanum*, *Rana esculenta*, *Xenopus laevis*, *Passer domesticus*, *Columba livia*, *Epimys norvegicus*.

The total number of animals studied was 90, using an average of 10 of each species, of either sex. The investigations took place during the summer months.

The animals were killed by decapitation. The brain was fixed in Bouin's or Helly's fixative, embedded in paraffin and sectioned serially at 7 μ. The sections were stained with Gomori's chrome hematoxylin phloxine by the modified method of BARGMANN, as well as with paraldehyde fuchsin, according to GABE.

Results

The glial cells staining electively with chrome hematoxylin and aldehyde-fuchsin have been found in every species examined by us. The cells are not uniform in shape, size and location; they can be divided into two morphologically distinct groups:

(*a*) The nucleus of one type of cell (Fig. 1 A) is round, in the rat the diameter averages 8 μ, the chromatin content is low. By the staining methods employed processes were seldom demonstrable on the cells. The cells occur in larger numbers periventricularly, under the ependyma. Their number decreases fast with the increase in the distance from the ependyma. This type of cell occurs in considerable numbers in the hypothalamus, but it is found in the epithalamus, too, usually around the third ventricle and under the ependyma of the aqueduct of Sylvius. In some species it occurs subependymally around the lateral and fourth ventricles, too.

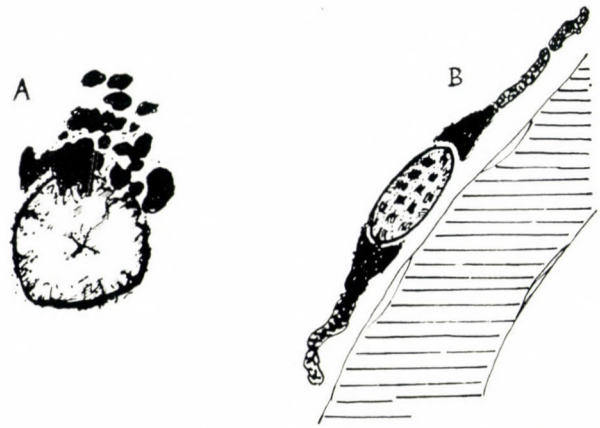

Fig. 1. Schematic representation of the "periventricular" *(A)* and the "perivasal" *(B)* types of Gomori-positive cells

This Gomori-positive type of glial cells is not found in the area of the neurosecretory cell groups, or, at the most, it is found in such areas in which the secretory nerve cells lie next to the ependyma.

(*b*) The other type of glial cells staining Gomori-positive (Fig. 1 B) is smaller than the first one. The cells have processes, are spindle-shaped or elongated. The nucleus, too, is smaller, oval rich in chromatin. In the rat the longitudinal diameter of the nucleus averages 6 μ, the shorter diameter about 3 μ. This type of cell occurs next to blood vessels, capillaries. The cells of this type can be detected in the periventricular gray matter, in the neurosecretory cell groups, but also in other, more distant areas of the brain, in the meninges, choroid plexus, peripheral ganglia, etc.; everywhere alongside the capillaries.

The morphological picture varies from species to species.

In *Rana esculenta* the glial cells of type one, with the big round, light nuclei, are found in the proximity of the cerebral ventricles (Fig. 2). In cross sections they make a single row of cells, immediately under the ependymal epithelium. They can be found subependymally in the area of the lateral

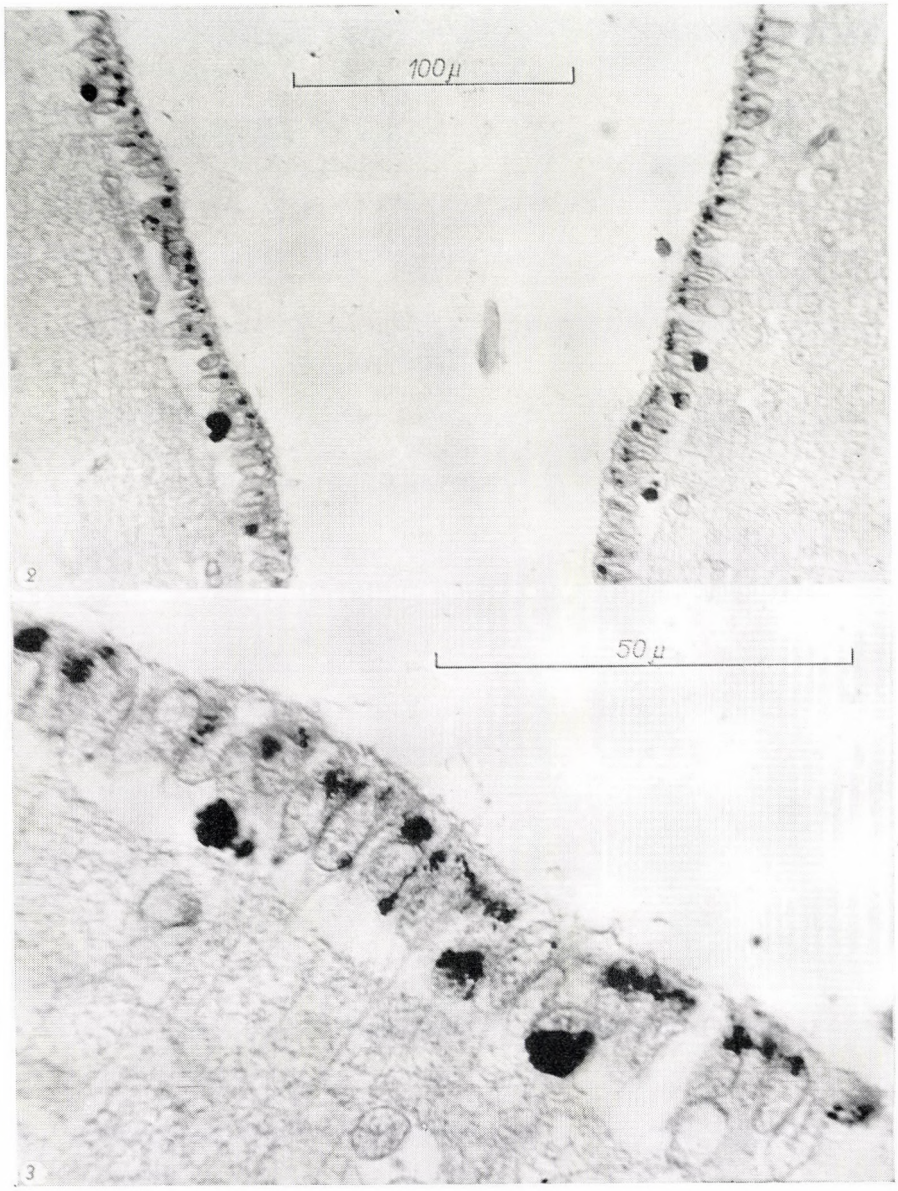

Fig. 2. Gomori-positive glial cells located periventricularly of *Rana esculenta*. Third ventricle. Paraldehyde-fuchsin stain. *Fig. 3.* Gomori-positive glial cells under secreting ependymal cells of *Rana esculenta*. Third ventricle. Paraldehyde-fuchsin stain

ventricles, optic lobe and fourth ventricle, too, but they occur in largest numbers in the area of the third ventricle. They are found always in areas in which the ependyma shows the signs of ependymosecretion, Gomori-positive secretion. The close correlation between these glial cells containing the Gomori-

positive granules and the secretory ependyma can be followed up clearly (Fig. 3). The correlation does not simply mean that they are found next to one another; we can namely find cells representing continuous forms of transition between the secreting ependymal cells and the Gomori-positive glial cells (Fig. 4). In the ependyma itself regular secretory cyclicity is observable: cells different in shape and filled in different measure with the Gomori-positive substance are found. At first, the ependymal cell is long, conical, contains a

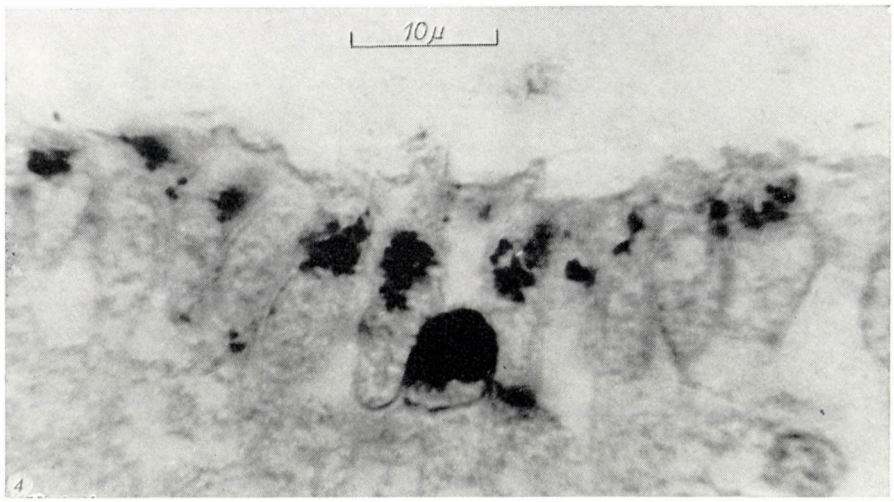

Fig. 4. Gomori-positive glial cell and ependymal cells filled in different measure with granules in *Rana esculenta*. Paraldehyde-fuchsin stain

few apical Gomori-positive granules, the nucleus is oval. The cell forms containing more granules are short, the nucleus is dislocated toward the base and is round. After that, the accumulated mass of granules fills out the cell as a whole. The nucleus is pressed against the base, the ependymal cell is rounded and lies next to the base of other cells. However, these rounded ependymal cells filled with secretion are absolutely identical already with the glial cells containing the Gomori-positive substance located subependymally.

The glial cells mentioned cannot be detected among the neurosecretory nerve cells (Fig. 5). In the area in which the neurosecretory nerve cells are found immediately under the ventricular ependyma, the ventricular ependyma exhibits in the frog no sign of ependymosecretion and no Gomori-positive glial cells can be found subependymally, either.

The cells of the other type, with the small, chromatin-rich nucleus and processes occur in the brain of the frog too. They may be found also near the secretory nerve cells, but always closely alongside blood vessels. Such Gomori-positive cells can be detected around the capillaries not only of the

hypothalamus, but of other areas of the brain as well. They occur in largest numbers in the meninges and in the choroid plexus (Fig. 6).

In *Xenopus laevis* we find similar morphological features.

In the brain of the newt *(Triturus cristatus)* we find cells similar to those occurring in the frog's brain. The subependymal Gomori-positive cells belonging

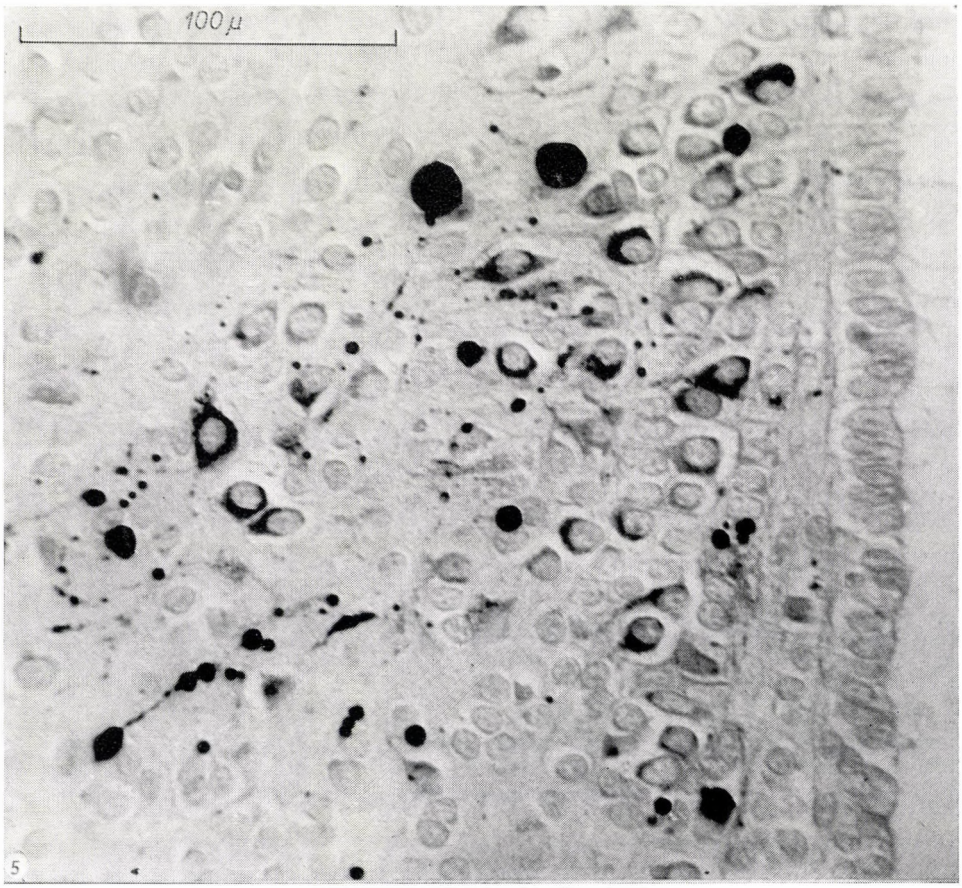

Fig. 5. Neurosecretory cells from the hypothalamus of *Rana esculenta*. No subependymal Gomori-positive glial cells are visible. Paraldehyde-fuchsin stain

to type one occur in smaller numbers. However, large numbers of the processed, perivascular cells are visible around the capillaries and veins (Fig. 7).

Essentially the same are found in the species
Triturus vulgaris,
Pleurodeles waltlii and
Amblystoma mexicanum.

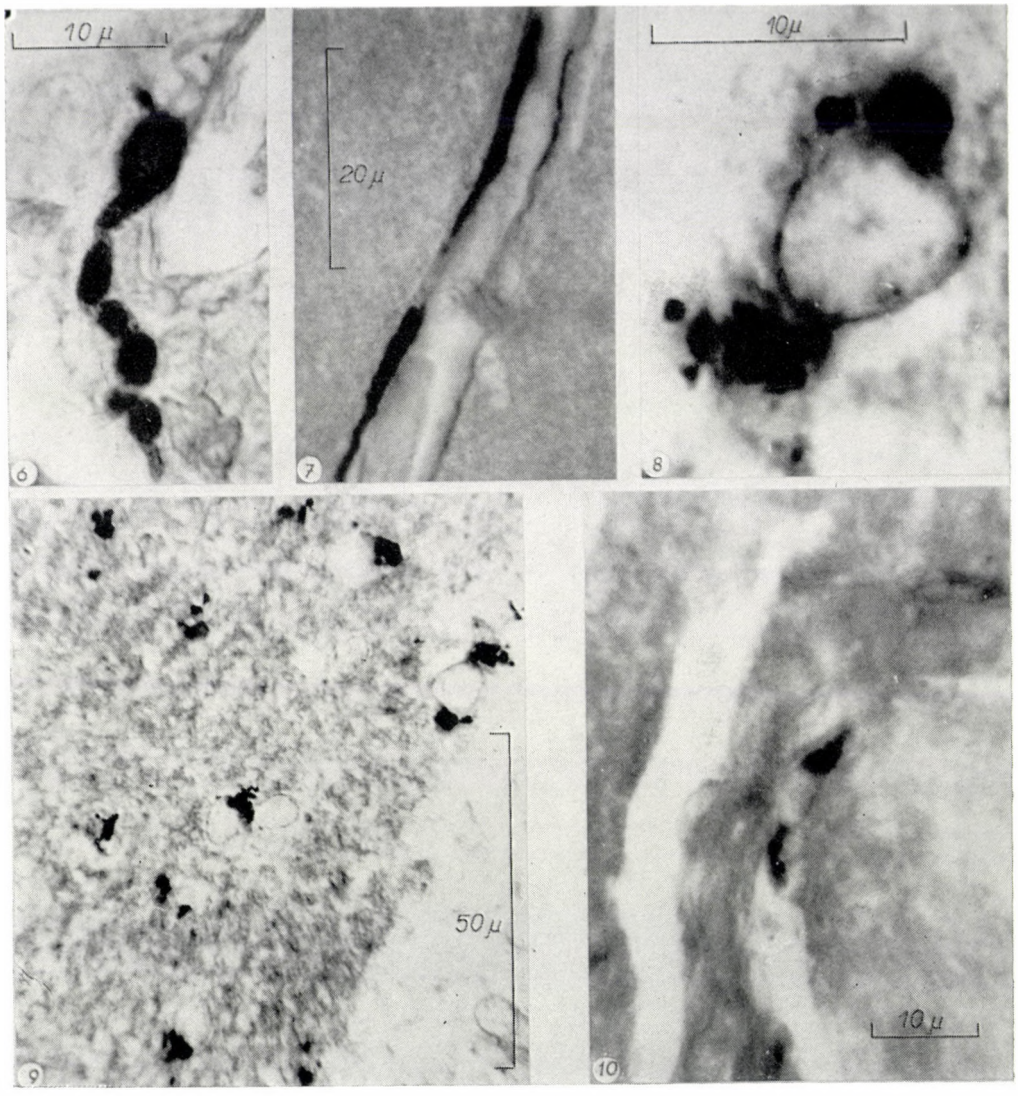

Fig. 6. Perivasal, multipolar Gomori-positive cell from the choroid plexus of *Rana esculenta*. Paraldehyde-fuchsin stain. *Fig. 7.* Gomori-positive cell adherent to capillary wall in the diencephalon of *Triturus cristatus*. Paraldehyde-fuchsin stain. *Fig. 8.* "Periventricular" Gomori-positive glial cell in the hypothalamus of the rat. Paraldehyde fuchsin stain. *Fig. 9.* Subependymal Gomori-positive glial cells in the hypothalamus of the rat, Paraldehyde-fuchsin stain. *Fig. 10.* Subependymal Gomori-positive glial cells with polymorphous nuclei in the hypothalamus of the rat. Paraldehyde-fuchsin stain

In *Passer domesticus* and in *Columba livia* many Gomori-positive glial cells are found around the third ventricle of the brain. The cytoplasm stains very lightly with the stain used by us. The Gomori-positive granules, which occur independently or in smaller or bigger groups, often draw out a few

short cellular processes. The other type of cell, too, is present in the birds we have studied. They are found perivasally in these species also.

The two types of Gomori-positive glial cells occur in the rat *(Epimys norvegicus)* too.

One type is bigger, the nucleus is round, measures about 8 μ, is poor in chromatin, processes are seldom visible on it (Fig. 8). It is found always near the ependyma, in the largest numbers around the third ventricle and the aqueduct of Sylvius (Fig. 9). It is characteristic that the nuclei, just like those of the secretory nerve cells and secretory ependymal cells are polymorphous.

The other type of cell containing Gomori-positive substance is smaller in size (Fig. 10), the nucleus is oval, rich in chromatin, measures about 3 $\times$ 6 μ. The intracellular Gomori-positive granules are small, much smaller than those of the other cell type and mostly fill the entire cell.

The cell form described occurs in considerable quantities in the rat's brain, especially next to the wall of the capillaries and smaller veins. They occur also in the hypothalamic neurosecretory nerve cell groups, but always in conjunction with capillaries. They can be found not only in the hypothalamus, but in other areas of the brain as well.

Discussion

On the basis of the questions outlined in the introduction, the considerations connected with the morphological patterns found in different vertebrates may be grouped as follows.

(*a*) From the point of view of comparative morphology it is significant that the glial cells containing the Gomori-positive substance occur in every species examined by us. In *Urodela* the number of periventricular glial cells is low and the Gomori-positive cells are represented first of all by the processed cells appearing as pericytes. This is probably due to the primitivity of the nervous system.

The periventricular glial cells appear in clear-cut form first in *Anura*, though they are even here rather simple, forming a single, scarce row of cells at the base of the ependymal cells.

In *birds*, just like in mammals, the periventricular periependymal Gomori-positive glia is already a well-defined periventricular system.

We have examined only rats from among *mammals*. On the other hand, similar glial cells have been described to occur in the dog by Noda [9]. Wislocki and Leduc [18] have studied mice, guinea pigs, as well as a Rhesus monkey and found also in them the periventricular Gomori-positive glial cells. It therefore appears that these glial cells exist from the amphibia to the highest mammals.

The Gomori-positive cells of species different in the grade of development are similar to one another.

(*b*) As to the more detailed morphology of the Gomori-positive glia, we can confirm the statements made by WISLOCKI and LEDUC [18]. The cells may, namely, be divided into two groups in the different vertebrates: bigger cells with few processes and round, light nucleus, on the one hand, and multipolar macrophage-like cells with oval nucleus rich in chromatin, on the other. These two morphologically distinct cell types appear in special areas. Those of type one always appear periventricularly, forming a kind of coat closely under the ependyma. Type two is found alongside the capillary walls.

The nucleus of the "subependymal" Gomori-positive glial cells is closely similar to that of the ependymal cells producing Gomori-positive secretion. The similarity manifests itself first of all in the size, shape and chromatin content of the nuclei. Besides, the nucleus of the glial cells is also markedly polymorphous (Fig. 11). Often it is quite lobated, intractions, glove-finger-like prolongations are visible in it. In this regard, too, the cell closely resembles the ependymosecretory cells, whose nuclei show a similar polymorphism [13]. The cells may contain variable quantities of granules. The granules are always rather big, bigger than those in the perivascular cells of type two; they are similar in size to the granules of the ependymosecretion, which is another evidence suggesting that a correlation exists between ependymosecretion and periventricular glia.

These cells differ from other, non-Gomori-positive glial cells not only in that they contain Gomori-positive substance, but also in that they possess absolutely special, well-defined form. Moreover, their site of occurrence is also special, inasmuch as they are found closely subependymally, forming a kind of periventricular system.

The other, the "perivasal" cell type is clearly distinct in morphology from the "periventricular" one; the cells of that type have small, oval nucleus, have processes or are spindle-shaped. Similar multipolar Gomori-positive cells have been found in the newt around the cerebral blood vessels by MAZZI [8], as well as in the choroid plexus of the axolotl by KAPPERS [6]. They think the cells are identical with mast cells. It is namely known that the granules of mast cells, too, are Gomori-positive [16]. However, it is also known that among similar cells also the melanocytes may give positive Gomori-reaction [3]. The melanocytes, too, occur perivascularly, and therefore we think that the present investigations and the data published in the literature cannot decide where the type two, perivascular Gomori-positive cells belong to. Further investigations, first of all detailed histochemical studies, will have to decide this problem.

(*c*) Finally, as to the relationship between the Gomori-positive glial cells and neurosecretions it has to be pointed out that there is a divergence of

opinion in this respect among the authors concerned with this problem. DIEPEN and co-worker [4] as well as NODA [9] claim that the granules of glial cells arise as a result of a phagocytosis of neurosecretory material. LÖFGREN [7] thinks the same. However, HILD [5] refuses to accept this view, starting from the fact that similar cells occur also outside the hypothalamus and that, unlike the neurosecretion, the granules stain even after treatment with fat solvents. BARRY [2] calls the granules of glial cells "granules denses" and thinks them to be different from neurosecretion. The above mentioned authors have not

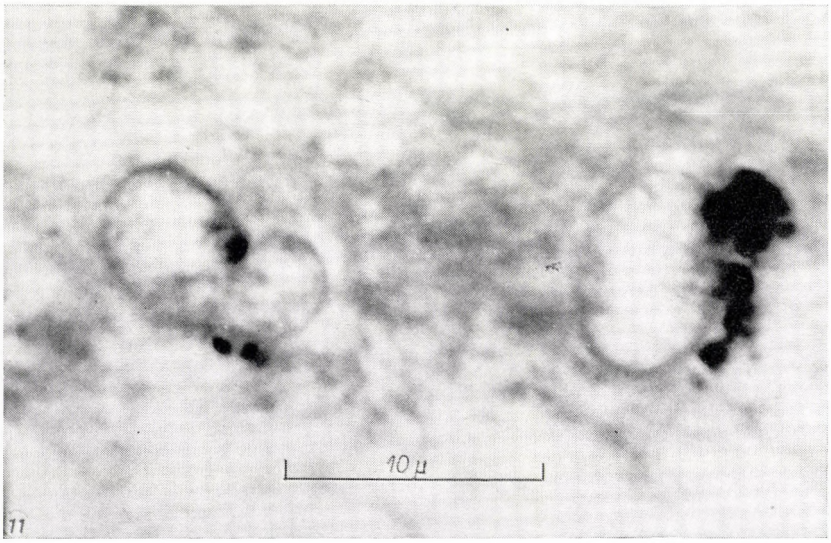

Fig. 11. Perivasal multipolar Gomori-positive cell from the hypothalamus of the rat. Paraldehyde-fuchsin stain

written about the two groups of Gomori-positive cells. WISLOCKI and LEDUC [17, 18] have analyzed the problem in detail and found that the Gomori-positive glial cells occur periventricularly and are definitely distinct from the perivascular Gomori-positive cells, which they called macrophage-like cells, but they have not written about the relationship between the neurosecretory system and the Gomori-positive glial cells.

In our present investigations we have found no morphological relationship between the type one "periventricular" Gomori-positive glial cells and the neurosecretory system.

On the other hand, a close correlation seems to exist between this cell type and the Gomori-positive secretory ependymal areas. In *Rana esculenta* the close correlation between these two areas is obvious. On the one hand, the cells are found in the areas in which the ependyma shows Gomori-positive secretion, and, on the other, every transitory cell-form exists between the ependymal

cells in different phases of secretory activity and the Gomori-positive glial cells under them (Fig. 5). From the pattern we think it likely that the ependymal cells — filled with secretory material and not evacuated — become rounded, penetrate under the epithelium and become transformed there to Gomori-positive glial cells. Accordingly, the granules of the glial cells are morphologically identical not with the neurosecretory, but with the ependymosecretory material.

Although Gomori-positive cells resembling glial cells do occur among the neurosecretory nerve cells, they are always found near capillaries and correspond to what we call type two of cells, perivasal ones with small, oval nucleus rich in chromatin, with small granules and processes. These Gomori-positive cells often occur directly between the capillary and nerve cell. It is conceivable that the granules of these cells originate from neurosecretion. However, a contradictory evidence is that they occur in similar or larger numbers also in other areas of the brain, in the meninges and in the choroid plexus. Further histochemical and experimental morphological studies are required to determine their true nature and their relationship to neurosecretory processes.

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EPENDYMOSEKRETION (EPENDYMALE NEUROSEKRETION)

V. Gomori-positives Material enthaltende Gliazellen und ihre Beziehung zur Ependymosekretion in verschiedenen Wirbeltieren

Gomori-positives Material enthaltende Gliazellen des Zentralnervensystems von geschwänzten und schwanzlosen Amphibien, Vögeln sowie Säugern wurden untersucht. Die Zellen können in allen von uns untersuchten Arten gefunden werden. Die Gomori-positiven Zellen kann man in zwei voneinander gut zu unterscheidende Gruppen einteilen: 1. „Periventrikuläre", wenig verzweigte Gliazellen, die ihren Platz unter dem Ependym nehmen und die über einen chromatinarmen, polymorphen Kern verfügen. 2. „Perivasaler" Zelltyp. Um Kapillaren und Venen herum, in verschiedenen Gehirngebieten in gleicher Weise erscheinende verzweigte Zellen mit chromatinreichem, ovalen Kern. Die periventrikuläre Gliazellart steht sowohl morphologisch als auch hinsichtlich ihrer Genese mit dem ependymosekretorischen System in Verbindung. Der perivasale Zelltyp ist in den neurosekretorischen Zellgruppen zu finden.

ЭПЕНДИМОСЕКРЕЦИЯ (ЭПЕНДИМАЛЬНАЯ НЕЙРОСЕКРЕЦИЯ)

V. Связь клеток глии, содержащих Гомори положительное вещество, с эпендимосекрецией у различных позвоночных

Исследовали клетки глии, содержащие Гомори положительное вещество, в центральной нервной системе хвостатых и бесхвостных земноводных, птиц и млекопитающих.

Клетки глии имеются у всех исследованные видов животных. Гомори положительные клетки можно разделить на две, хорошо различаемых группы:

1. «Перивентрикулярные» клетки глии, располагающиеся под эпендимой, имеющие мало отростков и бедные хроматином полиморфные ядра.

2. «Периваздальные» клетки. Эти клетки отросчатые и располагаются вокруг капилляров и вен в различных областях мозга; ядра их овальной формы и богаты хроматином

Перивентрикулярная разновидность клеток глии, как в морфологическом, так и в генетическом отношении связаны с системой эпендимосекреции. В группах нейросекреорных клеток периваздальный тип клеток обнаруживается.

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FUNCTION OF HETEROTOPIC SPINAL CORD SEGMENTS INVESTIGATED IN THE CHICK

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Synopsis

In chick embryos (Hamburger and Hamilton stages 15 and 16) the previously extirpated brachial cord segments were replaced by brachial, thoracic, or lumbo-sacral cord segments taken from other embryos of the same age. Innervation and function of the wings innervated by the grafted cord segments were investigated after hatching. The brachial segments of another chick can substitute for the extirpated brachial segments in every respect. Normal innervation and function of the wings had developed in such cases. Although the nerves of grafted thoracic segments innervated the wings, which became sensitive to pain, the musculature became completely atrophic and the joints ankylotic. Hypertrophy developed in the dorsal grey columns of the grafted segments, whereas the motor column exhibited certain signs of distrophy. Wings innervated by heterotopic lumbo-sacral segments showed moderate motility in the shoulder, perfectly synchronized with the movements of the legs on the same side. In spite of well developed and normally innervated musculature no coordinated movements in the other joints could be observed. The possible mechanisms underlying the immobility of the distal joints and the synchronized movements in the shoulder are discussed.

Introduction

A number of data support the assumption that coordinated limb movements can be controlled only by the appropriate limb innervating segments of the spinal cord in the newt [1, 3, 10, 15]. Supernumerary limbs, both in amphibia and birds, innervated by trunk segments remained motionless [9, 14], unless a single branch from the brachial plexus, however small, contributed to the limb innervation [4]. Quite recently direct experimental evidence was furnished by SZÉKELY [13] for the existence of a specific segmental apparatus in the cord at limb level, capable of eliciting coordinated function of the innervated limb. By transplanting brachial or lumbo-sacral segmental groups into the place of the previously excised thoracic segments and additionally grafting supernumerary limbs at the same level, he succeeded in demonstrating that besides perfectly coordinated stepping movements the participation of the supernumerary limbs in the stepping pattern was characteristic of the nature of the limb innervating heterotopic cord segments. As all of these experiments were performed on amphibia, it seemed promising to extend the investigations to birds whose more elaborate locomotion patterns and central nervous system

of histologically higher differentiation might contribute to the problem of the function of heterotopic spinal cord segments. We attempted, therefore, to replace the wing innervating brachial segments by thoracic and lumbo-sacral segmental groups in chicken embryos and to investigate the motility of such wings after hatching. An account of these experiments is presented in this paper. An abstract of this paper has been published elsewhere [12].

Method

The experiments were performed on the embryos of inbred strains of two local varieties of fowl (Hungarian Yellow and Speckled). The embryos were operated upon on the third day of incubation in stages 15 and 16 of HAMBURGER and HAMILTON [8]. The thoracic spinal cord segments of the donor were excised by the aid of the electromagnetic vibrating needle [5], then carefully cleaned from chorda and myotome material and after transferring in the acceptor egg, fitted into the place of the previously removed brachial (16 to 19) spinal cord segments. In another group of embryos the lumbosacral (23 to 29) segments were similarly grafted into the same place. To control the possible influence of the operation itself on wing function, the brachial segments of two embryos were interchanged in the third group of experimental animals. From a total of 226 operated embryos we succeeded in getting 17 viable hatched chicks, from which 4 bore thoracic, 11 lumbosacral segments on the place of the removed brachial segments, while 2 animals belonged to the control group. After hatching, the animals were kept alive from 2 weeks to 5 months. During this period motility of the wings was observed continuously and the most successful cases were recorded by cinematrography. After sacrification the animals were fixed in formol 1:4.

Innervation and musculature of the wings were studied partly by gross anatomical dissection and partly in microscopical sections stained with Bielschowsky's silver method. The histological investigation of the grafted cord segments has been performed also in Bielschowsky stained transverse sections.

Results

The general behaviour and growth of the operated chicks — disregarding the wings — was normal in the majority of cases. Reconstruction of the vertebral column and fusion of the grafted segments with the remaining cut

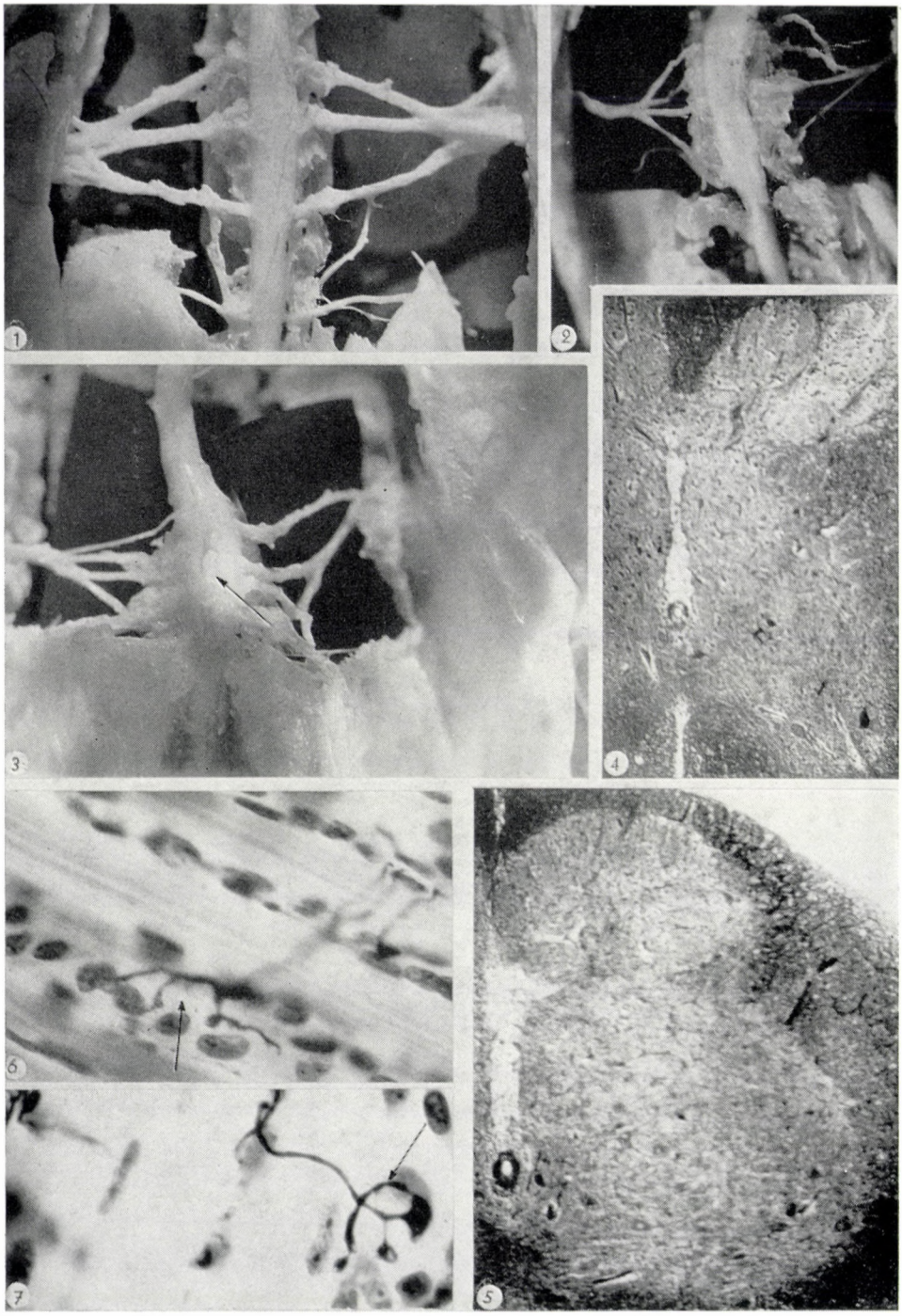
Fig. 1. Dissection finding concerning an animal in which the brachial segments had been interchanged between two embryos. Normal brachial plexus had developed from the 12th and 13th cervical segments and from the 1st thoracic segment. A strong branch from the 2nd thoracic segment contributes to the plexus formation. This chick was kept alive for 5 months.

Fig. 2. Dissection finding in thoracic segments grafted into the place of brachial segments. The spinal cord developed normally. The brachial plexus originates on the right side from the 11th, 12th, 13th cervical and from the 1st thoracic segment, while on the left side from the 12th, 13th cervical and from the 1st and 2nd thoracic segments. The chick was sacrificed 6 weeks after hatching

Fig. 3. Dissection finding in lumbo-sacral segments grafted into the place of brachial segments. The spinal cord developed normally and the characteristic glial swelling (arrow) was preserved. The branches of the brachial plexus emerged, as in normal animals, from four segments. The chick was sacrificed at the age of 5 months

Figs. 4 and *5.* Demonstrate the difference between the normal (Fig. 4) and a grafted (Fig. 5) thoracic cord of the same animal. The dorsal horn and the intermediate zone of the grafted segments are considerably enlarged and a reduction in number of ventral motoneurons appears to have taken place. Magnification 65 ×

Figs. 6 and *7.* Normal motor end-plates (arrow) have developed in the wing musculature, innervated by grafted lumbo-sacral segments



ends of the spinal cord was also complete, so that no sign indicated the site of operation. The function of the legs in 5 animals was more or less damaged, these chicks were mostly sitting on their belly and unable to perform normal locomotion. Small protrusions and deviations were found on their vertrebral column and the grafted segments were connected with the lower spinal cord through a somewhat narrowed region. No immunological reaction was detected even in animals which were kept alive for the longest period. The following characteristics were found in the several experimental groups.

1. In the two successfully hatched cases, in which brachial segments had been exchanged between two embryos, the function of the wings was

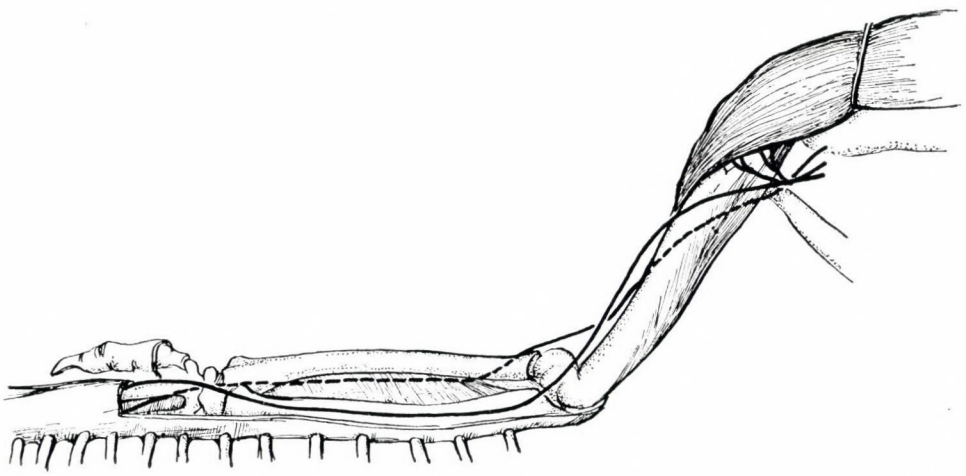


Fig. 8. The wing of a chick with thoracic segments grafted into the place of the brachial ones. The musculature of the free extremity has completely disappeared, save for a thin bundle at the place of the triceps. The median nerve (full line) and the radial nerve (partly broken line) follow the normal course of the wing nerves

indistinguishable from that in normal animals (Fig. 10). The dissection findings revealed complete fusion of the ends and normal plexus formation (Fig. 1). The arborization pattern of the peripheral nerves and the musculature of the wings, in as much as it could be traced under dissecting microscope, was exactly similar to those of an intact animal. Formation of regular end-plates was seen in the impregnated wing muscles. Heterotopic brachial segments can, therefore, substitute for the normal ones in every respect.

2. When the brachial segments had been replaced by the thoracic segments, the wings remained perfectly motionless. The joints became ankylotic and the wings were slightly retarded in growth. The chicks were unable to stand up when they happened to fall on the side or to be turned upside down, although locomotion by the legs was normal otherwise. The animals exhibited a remarkable sensitivity to painful stimulation of the wings, but

they could not localize the exact place of stimulation. The general locomotion of the animals was otherwise normal, save for some slight uncertainty in maintaining the equilibrium during certain strained movements as for instance jumping.

According to the dissection findings, the fusion between the grafted and orthotopic segments was complete (Fig. 2). Macroscopic cervical enlargement failed to develop and the nerves formed a plexus before entering the wings. In spite of the fact that five primordial thoracic segments had to be transplanted in order to fill in the gap left by the extirpated brachial segments, four segments developed from the grafted cord, and the innervation of the wing, like in intact animals, emerged from the 13 to 16th spinal nerves including a small branch from the 12th nerve. The nerves, however, were very thin, hardly exceeding the thickness of normal thoracic nerves. The spinal ganglia, as compared with the normal thoracic ganglia, were larger in size, owing to their relatively overloaded peripheric field, but they did not reach the size of normal brachial ganglia. The peripheric arborization pattern was suggestive of the normal wing (Fig. 8). The radial and median nerve could be well recognized; no side branches, however, were found in macroscopical preparation, owing, apparently, to the lack of musculature. This corroborates PIATT's [9] earlier observation that the arborization pattern is determined by the structure of the innervated organ. The histological pictures of the heterotopic segments revealed an enlargement, especially in the head of the posterior column, containing more neurons than orthotopic thoracic segments (Figs. 4 and 5). Considerable hypertrophy was found also in the intermediate zone. The number of motoneurons in the anterior column, on the other hand, was not increased. This finding is in accordance with HAMBURGER's observations [7], who has demonstrated that the motor column of an overloaded thoracic spinal cord does not change its size in contrast to the sensoric one which shows a clear gain in size correspondent to the peripheric load.

The musculature of such wings was atrophied. While the muscles of the girdle, although much smaller in size, more or less persisted no muscle was found on the free extremity, except for a small distrophic muscle bundle in the place of the triceps (Fig. 8). This bundle was present in every case.

3. The most interesting result of the experiments was the establishment of the function of wings innervated by heterotopic lumbo-sacral segments. A peculiar motility had developed which occurred always parallel with movements of the legs. When walking, the animals raised and adduced the wing to the shoulder joint (Fig. 12) exactly in time with the step of the leg on the same side. This movement of wing was of little excursion, about in the 15 to 20 degree range. Although the musculature of the free extremity was fairly well maintained and normally innervated (Figs. 6 and 7), the joints were mobile, no movements occurred in the other joints or they were so small as

to escape detection. The wings remained completely motionless under all circumstances that elicit the characteristic wing reflexes in intact animals. Letting fall down a chick provokes rapid flattering of the wings, or tilting around the longitudinal axis an extension of the wing on the side turned downwards and a flexion and adduction on the other. None of these responses appeared in the operated animals (Fig. 11). It was, on the other hand, interest-

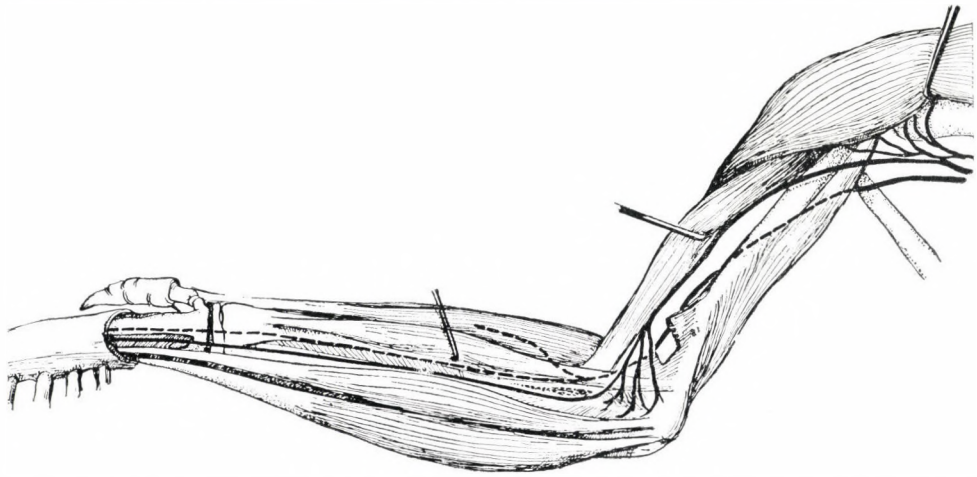
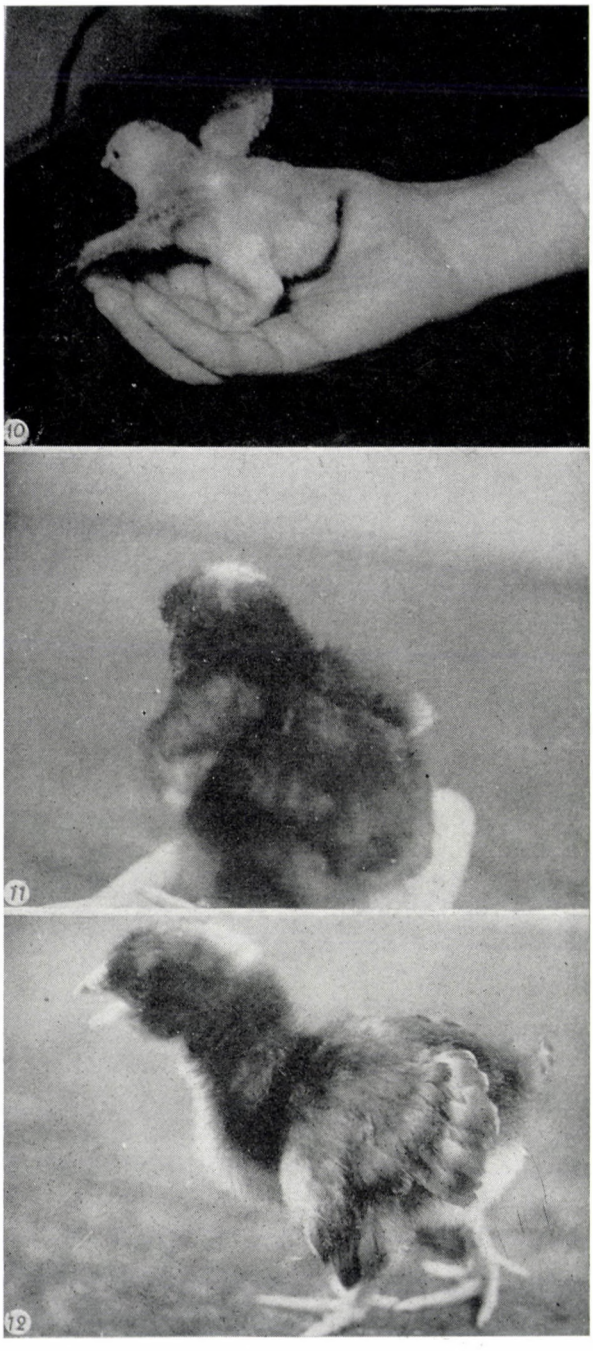


Fig. 9. The wing of a chick with lumbo-sacral segments grafted into the place of brachial segments. All muscles of the wing have developed, peripheral course and arborization pattern of the median (full line) and the radial nerves (broken line) are similar to those in the normally innervated wing

ing to note that when the chick was pecking and bending down for grains on the ground, all movements involving the hip joint were accompanied by the motions of the wings described above. From the study of the function of the wings the general impression was gained that the innervating lumbo-sacral segments on the place of the brachial segments did not react to central impulses addressed to the brachial cord, but were set into work simultaneously with the orthotopic lumbo-sacral segments.

Dissection studies revealed typical lumbo-sacral cord in the brachial region with the characteristic glial swelling in the rhomboidal sinus (Fig. 3).

Figs. 10, 11 and *12.* Show wing movement and posture in chicks with brachial spinal segments replaced homoplastically in early embryonic age. *Fig. 10.* The brachial segments have been replaced by the same segments of another embryo of similar age. Characteristic wing reflexes upon quick downward movement of the hand. *Fig. 11.* Lumbo-sacral cord grafted into the place of the brachial segments. Although wing muscles are well innervated and fairly well developed, there is no wing reflex during downward movement of the hand. *Fig. 12.* Similar case as in *Fig. 11.* with characteristic wing posture: somewhat extended and abduced. Simultaneously with each step the ipsilateral wing is clearly extended and abduced



From the seven primordial lumbosacral segments transplanted four cord segments developed and four nerves emerged which formed a plexus before entering the wing. The rearrangement of the heterotopic spinal cord in the number of segments both of lumbo-sacral and thoracic origin, was apparently influenced by the number of the neighbouring myotomes, as already shown by DETWILER [2] in the newt. The four plexus forming spinal nerves were mostly thicker than those of the heterotopic thoracic segments, but did not reach the thickness of the normal brachial nerves. Although the lumbar enlargement was present in brachial position as well, the histological examinations showed considerable loss of motoneurons as compared with the normal lumbo-sacral segments. The general histological appearence of the motoneurons left did not differ from that of the normal ones in any part of the spinal cord. The branching pattern of the peripheral nerves, as far as it could be traced under dissection microscope, was perfectly regular (Fig. 9) although the nerves were considerably smaller than in the normally innervated wings. The same held true for the muscular apparatus of these wings: all muscles were well developed, but their size did not reach, in general, that of normal wing muscles.

Discussion

As this investigation aims mainly at studying the function of heterotopic cord segments of different origin, the morphological findings are only briefly outlined, a more detailed discussion of myotrophic aspects being reserved for a following paper.

From the results the unequivocal conclusion can be drawn, that clear differences prevail in the functional capacity of different spinal cord segments, which are determined already in early embryonic life (Hamburger-Hamilton stages 15 and 16). Foreign brachial cord can substitute for the host's own brachial cord in every respect. As to the function of connecting higher and lower levels of the CNS, all segments seem to be equivalent. Thoracic segments are unable to perform whatever movement in the wing, which supports the several data available in literature [4, 10, 14]. The axons of thoracic motoneurons for some unknown reason cannot establish effective myoneural junction with wing musculature, which therefore soon undergoes complete atrophy, just as in the experimentally induced nerveless limbs of the chick [6]. Sensory nerves invading the wing can, on the other hand, promote pain and touch sensitivity. This difference is clearly represented by the distrophic motor column and the hypertrophic sensory columns in the histological pictures of the heterotopic thoracic segments.

Wings innervated by heterotopic lumbo-sacral segments are able to produce, if not complete, still considerable movement, synchronously with the

movements of the ipsilateral legs. There is no satisfactory explanation, as yet, to account for the incomplete movement in the otherwise normally developed and perfectly innervated wings. SZÉKELY [13] has reported a similar observation in the newt, according to which in forelimbs innervated by lumbo-sacral segments, although showing well coordinated stepping movements, the elbow moves only to that limited extent which can be observed in the knee of normal hindlimbs. But the adaptation of inappropriate segmental apparatus to the task of moving a limb is much better in the newt. It might well be that some higher plasticity of the segmental apparatus at the moment of the transplantation accounts for this difference. It must be considered, however, that the gross anatomical difference and consequently also the requirements of building up a highly specific nervous apparatus for coordinated limb movement are smaller between a newt fore- and hindlimb, than between wing and leg of the chick. This consideration is supported by the fact that both in the newt and the chick the movements in a limb, innervated by the inappropriate segmental apparatus, are better preserved in the proximal joints and progressively less in the distal ones, where the divergence between the two limbs becomes naturally larger and particularly explicit between wing and leg.

Another observation deserving some comment is the perfectly synchronous movement of the wings with the legs on the same side (Fig. 12). In similar experiments carried out on newts, SZÉKELY [13] could observe but an inclination to perform by the forelimbs movements synchronized with those of the hindlimbs, when the lumbo-sacral segments were substituted for the brachial segments. He suggested that the hindlimb character of the lumbo-sacral cord might have been not completely determined at the time of transplantation, and the grafted segments might have more or less adapted themselves to their new situation, resulting in forelimb trends of their function. Literature dealing with embryonic determination phenomena includes a large body of evidence suggesting that embryonic differentiation of most tissues, in general, is earlier determined in the chick than in the newt. The dissimilarity between these and Székely's findings can, therefore, be explained by assuming that the lumbo-sacral cord, being in the chick definitely determined at the time of transplantation, can maintain the hindlimb character in its function. Perfect synchronisation of movements in both limbs and the fact that the wings remain motionless under circumstances that in normal animals evoke wing responses, suggest furthermore that the grafted lumbo-sacral segments can somehow select from impulses descending from higher centres to answer only to those which are addressed to the normal lumbo-sacral segments. Trying to interpret such selective responses of the grafted segments, one may think of two alternatives: (i) The motoneurons of the grafted and the normal lumbo-sacral segments might establish connections with exactly the same descending neuron systems, and these specific neuronal connections might rise to the synchronous move-

ments in the extremities. The establishment of the specific connections might be regulated by some specific properties, claimed by WEISS and SPERRY [15, 11] to exist in the neurons, which would allow the development of functioning synapses only between sets of certain particular neurons having gained during neurogenesis qualitative specification to match each other. (ii) The alternative is that certain parts of the CNS have the capacity to analyze different impulse patterns and answer only to the appropriate ones, as suggested in earlier works from this Department [14]. The limb segments of the spinal cord might have some property of this kind, probably on the grounds of a peculiar structural arrangement of neurons in the segmental apparatus. Further experiments are necessary to analyze these problems.

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DIE FUNKTION HETEROTOPISCHER RÜCKENMARKSEGMENTE BEIM HÜHNCHEN

Bei Hühnerembryonen der Stadien 15–16 (HAMBURGER–HAMILTON) wurden die operativ entfernten Brachialsegmente des Medullarrohres durch brachiale, thoracale und lumbosacrale Medullarrohrabschnitte gleichartiger anderer Hühnerembryonen ersetzt. Innervation und Funktion der durch solche Implantate versorgten Flügel wurde nach dem Ausschlüpfen der Hühnchen untersucht.

Brachialsegmente eines anderen Embryos können die entfernten Brachialsegmente des Wirtstieres in jeder Beziehung ersetzen. Innervation und Funktion der Flügel erwiesen sich in solchen Fällen als normal.

Obwohl an Stelle des entfernten Brachialteils implantierte thoracale Segmente die Flügel innervierten und dieselben schmerzempfindlich waren, war ihre Musculatur vollkommen atrophisch und es entwickelte sich eine Ankylose der Gelenke. Die Hinterhörner der implantierten Rückenmarkssegmente zeigten wohl eine deutliche Hypertrophie, die Vorderhörner waren dagegen atrophisch.

Flügel, die durch implantierte lumbo-sacrale Segmente innerviert wurden, zeigten lediglich im Schultergelenk kleinere stets mit den Schreitbewegungen der gleichseitigen unteren Gliedmassen synchronisierte Bewegungen, aber keinerlei für die Flügel sonst typische Bewegungen und Reflexe. Trotz gut ausgebildeter und normal innervierter Flügelmuskulatur waren in den anderen Gelenken keinerlei koordinierte Bewegungen nachweisbar. Erklärungsmöglichkeiten dieser Befunde werden diskutiert.

ИССЛЕДОВАНИЕ У ЦЫПЛЯТ ФУНКЦИИ ГЕТЕРОТОПИЧЕСКИХ СПИННОМОЗГОВЫХ СЕГМЕНТОВ

В зародышах цыплят (стадии 15—16 по *Гамбургеру* и *Гамильтону*) предварительно удаленные плечевые спинномозговые сегменты были замещены плечевыми, грудными, или пояснично-крестцовыми сегментами спинного мозга, взятыми от других зародышей одинакового возраста. После вылупления из яйца исследовались иннервация и функция крыльев, иннервированных от пересаженных сегментов спинного мозга.

Плечевой сегмент другого цыпленка может заменить удаленный плечевой сегмент во всех отношениях. В таких случаях развивалась совершенно нормальная иннервация и функция.

Хотя нервы пересаженных грудных сегментов разветвлялись в крыльях, которые стали чувствительными в отношении болей, мышцы стали совершенно атрофическими и суставы анкилозными. В дорсальном сером веществе пересаженных сегментов наблюдалась гипертрофия, тогда как в двигательном нерве имелись определенные признаки дистрофии.

В крыльях, иннервированных от гетеротопических пояснично-крестцовых сегментов, наблюдались умеренные движения плеча, совершенно синхронные с движениями ног той же стороны. Вопреки хорошо развитой и нормально иннервированной мускулятуры не удалось установить координированных движений других суставов. Обсуждается предполагаемый механизм анкилоза дистальных суставов и синхронных движений в плечах.

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THE AMOUNT OF MITOCHONDRIA IN THE CELLS OF THE GROWING ZONES OF LUPINE ROOT

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Synopsis

The amount of mitochondria in the three growing zones (meristem, zone of cell elongation and the zone of root hair development) of lupine roots was determined by direct counting on thin microscopic sections or on the basis of N content of the mitochondrial fraction obtained by differential centrifugation. The amount of mitochondria changes in the root cells as they pass the various phases of growth. Maximum values are found in cells which have just completed the elongation, while minimum number is obtained for meristematic cells. The observed changes might in part account for the development of gradients of certain physiological characteristics in the lupine root.

Introduction

The close relationship of growth and absorption in the root system is now firmly established. This explains recent interest in the metabolism of growing zones of the root and in the elucidation of their role in uptake and translocation of inorganic substances. These studies supply a picture on the metabolic changes in cells on different stages of growth, on the one hand, and may characterize the functional development of the root as the organ of uptake and translocation of substances, on the other. Several studies [18, 19, 20] revealed certain differences between the cells of growing zones of the root as to the accumulation of dry matter, protein and nucleic metabolism, respiration, activity of certain oxidases, etc.

The recognition of the almost exclusive role of mitochondria in release and transformation of energy in living cells induced the study of distribution and activity of these organelles in the growing zones of root.

The present paper involves data on quantitative determination of mitochondria in cells of growing zones in lupine root. Authors are fully aware of the fact that mitochondrial number alone is not an exclusive criterion of the energetic capacities of the cells. The latter can be determined only if the potencies of the mitochondria and their working capacity under experimental conditions are known.

We assume, however, that the results of the first phase of our work represent certain interest in view of the paucity of reliable quantitative data on the mitochondrial number of plant cells.

The number of mitochondria in cells of different animal organs has been estimated in several papers [3, 4, 5, 16, 23]. Numbers ranging from 1400 to 2500 per cell were found in animal tissues; the half life period of the organelles was 5 to 10 days [10]. As to plants, more mitochondria were found in young cells as compared to old cells [21, 22]. The number of mitochondria per unit area remains relatively constant even when the cell volume increases [6, 22]. This observation permits the assumption that the mitochondrial number might increase as the elongation and differentiation of cells proceed. A similar conclusion is suggested by data concerning the protein content in the mitochondrial fraction of cells of different age [1, 2, 9, 15]. In root tip cells of four grass species the number of mitochondria per cell area was estimated to be 40 to 100 [6, 7]. Electron microscope and biochemical studies on mitochondrial development in maize root tip [15] revealed several changes of mitochondria during cell growth and differentiation. Immature mitochondria of low biochemical activity are transformed into differentiated active organelles in the cells which have just completed elongation. Most probably destruction of the structure and decline in the activity of mitochondria occurs in mature cells. The present data on percentual ratio of mitochondrial to total cell protein content also suggest that changes occur in mitochondrial number, structure and functional activity.

Materials and methods

Changes in the mitochondrial population of growing cells were studied in the present work by means of direct counts on permanent slides and by determination of total and protein nitrogen in the mitochondrial fraction obtained from the growing zones of lupine roots. Five to eight day old seedlings of the lupine variety Blue Nemtchinovska were used. The plants were grown in 1/5 Knop solution of pH 6.2. For direct counts root tips were cut into zones, fixed in Regaud and Levitsky [11, 21]. 3 μ thick cross sections were cut, stained with Heidenhain haematoxylin and the mitochondria counted in serial sections. The data were obtained by counting in 10 roots. In addition, mitochondrial counts were made on sections of living roots after reaction for succinic dehydrogenase [6]. The mitochondrial fraction was prepared by differential centrifugation (35 min. 17000 rpm) in an MSE refrigerated centrifuge. The preparation was performed in a refrigerated room at $0+3°$ C. The material was homogenized and centrifuged in sucrose solutions made up with phosphate buffer of pH 7.4 to which 5.10^{-4} M versene was added. 0.88 M sucrose was used for the division zone, whereas 0.25 M solution for the stretching zone and for the zone of root hairs. Total and protein nitrogen were determined according to the method of LUBOCHINSKY and ZALTA [14]. Cell number in the growing zones has been counted according to BROWN's method [8].

Results and discussion

The number of mitochondria in the cell areas on the section of the cell is rather constant (Table 1). Data obtained with plant sections (42 to 58 [6, 7]) and in sea urchin larves (10 to 30 [12]) are in good agreement with the

present observations. Table 1 shows the mitochondrial number in cells of tissues in the zone of cell division (0 to 2 mm) and of cell elongation (2 to 7 mm).

Table 1

Mitochondrial number per cell in the tissues of the first cm of lupine root (8 day old seedlings)

Tissue	Number of mitochondria		
	on the section in one cell	the same on the basis of tetrazolium reaction	in one cell
0—2 mm			
root cap	12.8 ± 0.5	15.6 ± 0.9	864 ± 49
meristem	22.0 ± 0.1	24.6 ± 0.8	149 ± 1.3
2—7 mm			
cortex	35.9 ± 0.7	35.1 ± 1.2	1176 ± 38
epidermis	31.2 ± 1.5	29.9 ± 1.5	936 ± 43
endodermis	27.3 ± 2.1	—	810 ± 41
pericycle	25.3 ± 2.1	—	800 ± 37
cell of central cylinder (plerome)	15.0 ± 0.4	—	1696 ± 58

The mitochondrial population in an average meristematic cell is about 150. This number increases parallel to the growth of the cell and differentiation of primary tissues. A considerable number of mitochondria is found in the elongating cells of vascular bundle which will form later where sieve tubes of the phloem are already developed. The counting in permanent mounts of the root hair zone encounters difficulties because the cytoplasm is located at the cell wall and no reliable counts can be obtained. The mitochondrial number in cells of this zone may be assessed on the basis of the total and protein nitrogen content in the mitochondrial fraction (Table 2).

Table 2

Nitrogen content of the mitochondrial fraction separated from growing zones of lupine seedlings (mg 10^{-9} per cell)

Growing zone	Age			
	5 days		8 days	
	total N	protein N	total N	protein N
division	40.2	30.2	24.5	19.0
elongation	92.1	60.1	75.6	44.7
root hair development	73.2	34.9	76.1	31.8

The values shown in Table 2 are means of 8 experiments. They show a decreased mitochondrial protein content in the dividing and elongating cells and no changes in the mature cells.

The protein nitrogen content of the mitochondrial fraction depends on the age of the cells. It attains a maximum level in the cells of the zone of elongation and decreases again in the mature cells of the zone of root hairs.

At the same time there is an increase of non-protein nitrogen in the mitochondrial fraction of the zone of root hairs which is probably due to the destruction of the internal structure of mitochondria. The increase of mitochondrial nitrogen in the zone of elongation might depend on an increase in the number of mitochondria, on the on ehand (Table1), and on their increased internal differentiation, *i.e.* on the increase in number of their cristae, on the other. In mature cells in the zone of root hairs the number of mitochondria probably decreases with parallel changes in their structure and activity. When the total nitrogen content of the mitochondrial fraction is expressed as per cent of the total nitrogen content of all fractions a rather constant value is obtained which does not depend on the age of the cells and roots (Table 3). These values fall at the same time within the range reported in the literature for different animal tissues (26.9 to 34.0 per cent).

Table 3

Total nitrogen content of the mitocdondrial fraction in per cent of total nitrogen content of all fractions studied

Growing zone	Age of seedlings in days	
	5	8
division	35.1	37.9
elongation	38.9	40.7
root hair development ...	32.8	34.5

A comparison of the present findings with data published earlier [13] shows a remarkable agreement in the total protein content per cell in rat liver and in the two first growing zones of lupine root.

The results obtained in the present study must be taken with great caution in view of the great difficulties connected with direct counting and of the uncertainty of the completeness of the mitochondrial fraction isolated by means of differential centrifugation. However, a comparison of the present data with results obtained with animal cells show that our results are real. *E.g.* the mitochondrial population in cells of cortex or of the central cylinder is only slightly less than in animal cells. There is a very close agreement in the mitochondrial nitrogen content per cell. In view of the ideas outlined above the results obtained in this work seem to be quite real.

Accordingly a gradient of mitochondrial number was found in the growing zones of the lupine root. The number of mitochondria changes in the cells as they pass the various phases of growth. Maximum values are found in cells which have just completed the elongation while these values somewhat decline in the cells of the root hair zone. Minimum number is obtained for meristematic cells. The observed changes might in part determine the development of certain physiological gradients in the lupine root.

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ANZAHL DER MITOCHONDRIEN IN DEN ZELLEN DER WACHSTUMSZONEN DER WURZEL VON LUPINEN

Die Anzahl der Mitochondrien in den Zellen der drei Wachstumszonen (Teilungszone, Streckungszone und Zone der Wurzelhaare) wurde durch Zählung in dünnen Mikrotomschnitten und auf Grund des Stickstoffgehaltes der durch Differenzialzentrifugierung erhaltenen Mitochondriumfraktion bestimmt. Es wurde eine Veränderung der Anzahl der Mitochondrien in den Wurzelzellen festgestellt, welche darauf zurückzuführen ist, dass die Zellen nacheinander zu den aufeinanderfolgenden Zonen gehören. Die grösste Anzahl wurde in den Zellen gefunden, die soeben die Streckung vollzogen haben, während die kleinste in den Meristemzellen. Die nachgewiesenen Veränderungen gehören nach der Meinung der Verfasser zu den Faktoren, welche das Auftreten von Gradienten in bestimmten physiologischen Eigenschaften der Lupinenwurzel bedingen.

О КОЛИЧЕСТВЕ МИТОХОНДРИЙ В КЛЕТКАХ ЗОН РОСТА КОРНЯ ЛЮПИНА

В статье приводятся результаты количественного распределения митохондрий в клетках трех зон роста корня люпина (меристема, зона растяжения и зона корневых волосков). О количестве митохондрий авторы судили по данным непосредственного подсчета их на тонких микротомных срезах и по количеству азота во фракции митохондрий, полученной дифференциальным центрифугированием. Авторы установили, что при прохождении клеткой корня отдельных этапов роста количество митохондрий в ней меняется. Число митохондрий максимально в клетках, закончивших растяжение, минимально — в клетках меристемы. Обнаруженные изменения в клетках зон роста корня люпина, по мнению авторов, являются одним из факторов, обуславливающих возникновение градиента ряда физиологических свойств в них.

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$^{14}CO_2$ INCORPORATION IN NORMAL AND ALBINO CORN LEAVES AT DIFFERENT LIGHT INTENSITIES

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Synopsis

The $^{14}CO_2$ assimilation of normal and albino corn seedlings was compared at different light intensities. It has been established that more $^{14}CO_2$ was fixed by the alcohol soluble and insoluble fractions of albino leaves at 5 and 100 lux intensities, than by the corresponding fraction of the normal leaves. At higher intensities (1000, 10 000 lux), however, it was found that the fractions of the normal leaves were labelled much more intensively than the corresponding fractions of the albino leaves, which at these intensities already exhibited a decreasing trend. The ^{14}C fixation maximum of albino leaves appears between 100 and 1000 lux, which is clearly illustrated also by the initial increase and subsequent destruction of pigment content.

By the quantitative distribution of ^{14}C among the alcohol soluble compounds it may be seen that light, besides affecting the extent of ^{14}C fixation, largely influences the further pathway of carbon too. As light intensity is raised, the ^{14}C contents of alanine, serine, glycine, and aspartic acid increase in normal leaves, whereas in albino leaves the ^{14}C fixation over 100 lux is shifting towards the organic acids. These data show that the difference between the light enduring capacity of normal and albino leaves influences the further mechanism of $^{14}CO_2$ fixation.

Introduction

The efficiency of plant photosynthesis, and the distribution of CO_2 incorporated into the different compounds, is greatly influenced by the wavelength and intensity of light. This effect may assert itself differently in the various photosynthetic processes. Light intensity also plays a role in the formation of the pigment content of the leaves which is connected to the relation between their synthesis and decomposition [27, 45, 46]. To maintain the dynamic equilibrium a light intensity is needed, at which pigment content and/or photosynthetic activity of the pigment are at a maximum. There is an activity decrease, at illumination appreciably stronger than optimum, which may be followed by an increasing pigment decomposition [52, 31, 50]. This process occurs even at lower light intensities in the case of very light sensitive albino plants [22, 48].

Strong illumination has an effect on CO_2 assimilation processes too. In such cases CO_2 incorporation partly or completely discontinues and "dark fixation" dominates [47].

Light intensity changes — besides influencing the extent of CO_2 incorporation — may influence the further pathway of oxygen and carbon also in leaves having normal pigment content.

BASSHAM [6] has shown that during normal assimilation most of ^{14}C (70—85%) is fixed when ribulosediphosphate carboxylase is present. Experiments conducted with etiolated bean leaves [10] showed a fast incorporation of $^{14}CO_2$ at 20,000 lux light intensity and there was of ^{14}C into the leaf pigments too. Illumination, first of all, stimulates α-alanine, serine, and glycine synthesis. The start of the β-carboxylation of phosphoenolpyruvic acid is indicated by increased activity of aspartic and glutamic acid.

Proteins are the first to be labelled at weak illumination, while at strong illumination — on account of the increased activity of the Calvin cycle — the synthesis of carbohydrates predominates [40, 41]. When illuminated with light of different wavelength, it was found that a higher proportion of CO_2 was incorporated into the amino acids of leaves kept in bluish-green light; in red light, however, the formation of carbohydrates comes into prominence [17, 42].

The synthesis of organic acids continues [20, 21] both in light and dark, the Krebs-cycle, however, is inhibited in intensely illuminated leaves, and CO_2 fixation is of less significance [4].

CO_2 assimilation tests conducted with ^{14}C, show that the wavelength and intensity of light influences the distribution of activity with in the molecule. CAYLE and EMERSON [13] found that in alanine the position of ^{14}C synthetized in blue and red light was similar; in glycine the amount of ^{14}C in α position was 21% in red light, and 42% in blue light. BRADBEER [12] determining the proportion of labelling in the C-1 and C-4 atoms of malic acid formed during assimilation, pointed out that CO_2 incorporation into the C atoms of malic acid was different depending upon light intensity. The metabolic differences referred to arise from the fact that light intensity is diversely involved in the various paths of metabolism [32, 56].

Shade-plants are more sensitive to light of different wavelength and energy [58], which is also shown by the relation between the distribution of synthetized CO_2 and light tolerance.

The aim of our work was to examine, which way the CO_2 assimilation of the genetically extremely light sensitive albino plants differs — at various light intensities — from the similarly treated normal leaves.

Materials and methods

Our experiments were made with the offspring of *Zea mays* heterozygote for albino mutation. Our albino strain synthetizes protochlorophyll in the dark to an extent approaching normal, which on light becomes transformed into chlorophyll-a, and chlorophyll-b. The carotenoid synthesis of the strain is abnormal, since it accumulates a partly saturated carotenoid: ζ-carotene [18]. The homozygote recessive albino phenotype can be distinguished easily, by the pale yellow coloured endosperm of kernels, from the homozygote dominant and heterozygote normal grains. The strain was inbred at the Alsógöd Biological Station of this University. The corn seeds, used for the $^{14}CO_2$ assimilation tests, were germinated in the

dark at 27° C. The 3 days old seedlings were placed in the $^{14}CO_2$ atmosphere, then kept at light intensities of 5, 100, 1000, and 10,000 lux, supplied by a 500 watt low pressure Xenon bulb. The $^{14}CO_2$ atmosphere was produced in a 4-compartment case of plexiglass [23], in which the light intensities mentioned above could be secured under otherwise similar conditions. A small dish was placed into the lower, inner meeting point of the compartments where 20 mg Ba $^{14}CO_3$ having an activity about 900 μc, and methyl red indicator showing the endpoint of the liberation and fixation reaction were placed.

The shoots of the seedlings were disposed in the compartments, in small dishes containing phtalate buffer (0.005 M phtalic acid; pH 4.8). Subsequently, the case was hermetically sealed. After producing a low vacuum, CO_2 was released by an excess of lactic acid made available from a ground glass burette. After 48 hrs. $^{14}CO_2$ absorption the CO_2 content of the atmosphere was fixed by an excess of $Ba(OH)_2$. The leaves were homogenized with sand and some $MgCO_3$ in acetone, then the lipid soluble components were removed by ether-extraction [32, 33]. The soluble components were dissolved from the residue by repeated alcoholic extraction, and were added to the water-acetone extract. An aliquot was brought from the total pigment as well as from the alcohol soluble fractions, on a small aluminum dish and the activities were measured after during. The total activity of the protein fractions were measured by hydrochloric hydrolysis [43].

To evaluate each of the components of the alcohol soluble fractions, a quantity of the material corresponding to an activity of 5×10^5 tpm. was taken up on a Whatman No 1. paper washed previously in oxalic acid. The soluble carbohydrates, amino and organic acids were separated by two-dimensional chromatography using BENSON's [9] solvent system (1. water-saturated phenol, 2. n-butanol-propionic acid-water 100/50/70). The chromatograms, when run, were placed on "Agfa Laue" X-ray films and exposed for 10 days. The developed film showed the position of the ^{14}C labelled materials. For quantitative evaluation — on the basis of comparison with X-ray films — the constituents were eluted by hot water from the paper, then placed on a small plate and the activities were measured by 2π geometry gas-flow GM tube. The characterization of the various spots was based on R_f values and map, as well as information by special developers.

Results and discussion

Table 1 shows the ^{14}C activities incorporated into the lipid soluble fractions (pigments, other lipid materials) of leaves kept in $^{14}CO_2$ atmosphere for 48 hrs at different light intensities.

From Table 1 it may be seen that the ^{14}C content of the lipid soluble fraction of normal leaves increases with growing light intensity. At strong illumination (10 000 lux) the activity is significant. There is an increasing trend of activity in albino leaves at low light intensities while at stronger

Table 1

The distribution of $^{14}CO_2$ fixed by the lipid soluble fractions of normal and albino leaves of corn subjected to different light intensities
(10^3 cpm/g fr. w.)

Light intensity (lux)	5		100		1000		10,000	
material	normal	albino	normal	albino	normal	albino	normal	albino
10^3 cpm/g	5.6	4.7	5.7	5.2	16.0	5.1	74.0	4.8
10^3 cpm/lux ...	...	...	0.06	0.05	0.02	0.005	0.007	0.0005
a/n	0.8		0.9		0.3		0.07	

Table 2

The quantity of $^{14}CO_2$ fixed by the alcohol soluble compounds of normal and albino leaves at different light intesities
(10^3 cpm/g fr. w.)

Light intensity (lux)	5		100		1000		10,000	
Material	normal	albino	normal	albino	normal	albino	normal	albino
10^3 cpm/g	44.0	54.0	34.0	55.0	310.0	59.0	5832.0	58.0
10^3 cpm/lux ...	....	....	0.3	0.6	0.3	0.06	0.5	0.006
total fix.%	0.96	0.95	1.10	0.61	4.18	0.53	11.20	0.70
a/n	1.2		1.6		0.2		0.01	

illumination it drops to the level corresponding to the value of the dark variant. $^{14}CO_2$ incorporation is the most intensive at 100—1000 lux intensities. The differences, between the amounts of ^{14}C incorporated into the lipid soluble fractions of normal and albino leaves, are illustrated by the activity increase (cpm/lux) related to the different light intensities and/or by the albino/normal, (a/n) ratio. The cpm/lux values are the same in normal and albino leaves at 100 lux intensity. From this it may be concluded that, at this light intensity, the pigment synthesis of the albino leaves is also normal.

At 1000 lux light intensity the cpm/lux value hardly differs from the value of the 100 lux variant; in the albino leaves, however, it is about 1/10 of the original value. This tendency is more explicit at 10 000 lux, in so far as the cpm/lux value in the normal leaves drops to 1/10 of the cpm/lux value of the variant kept at 100 lux; in the albinos this drop may amount to two orders of magnitude. At this light intensity the albino leaves are practically bleached.

The a/n quotient is the highest at 100 lux light intensities, then — because of the decomposition of the albino leaf pigments, and mainly due to intensive increase of the pigments in the normal leaves — it rapidly decreases with the increase of illumination.

In our previous work we have stated that there was a difference of about two orders of magnitude between the light tolerance of the normal and albino leaf pigments [22]. Considering the relatively high activities at 10,000 lux, one may arrive at the conclusion that the high light intensity in albino leaves, though increasing the decomposition of the pigments, only slightly damages the pigment synthetizing system. Concerning the pigment destruction in the strongly illuminated mutant plants, the hypothesis seems probable that this process is connected with the increased light sensitivity of the caroteneids of the albino leaf pigments [18].

The distribution of ^{14}C incorporated into the alcohol soluble fraction containing amino acids, sugars, and organic acids of normal and albino leaves kept at different light intensities is shown in Table 2.

It appears from Table 2 that the ^{14}C content of the normal leaves, established at 5, or 100 lux light intensities, was nearly the same, and lower than the corresponding values of the albino variety. At 1000 and 10,000 lux the activity is substantially higher.

The activity change is slight in the albino leaves with a maximum value at 100 lux. At 10,000 lux it begins to slightly decrease.

At 100 lux the cpm/lux value of the albino leaves is the double of the normal ones; at 1000 lux this value decreases tenfold, while there is no change in the normal leaves. At 10,000 lux the cpm/lux value of the albino leaves decreases again tenfold, while in the normal leaves the decrease amounts to 60%, as compared to the values obtained at the previous light intensities. A comparison of the a/n ratios reveals that, at 100 lux intensity, there is a much stronger fixation of CO_2 by the albino leaves, than by the normal. This ratio largely decreases at higher light intensities, on account of the more intensive $^{14}CO_2$ fixation of the normal leaves. This intensive fixation is significant, even as compared to the total fixed $^{14}CO_2$ (11.2%), which may be mainly correlated with the activity decrease of the "solid" fraction. The activity shift toward the alcohol soluble compounds may be explained by a drop in the formation, or an increase in the decomposition of the insoluble compounds.

Table 3 shows the activities of ^{14}C fixed by the insoluble fractions made up by proteins and different polysaccharides. There was a gradual increase in activity — apart from the low value at 100 lux — in the leaves of normal

Table 3

The quantity of $^{14}CO_2$ fixed by the alcohol insoluble fractions of normal and albino leaves at different light intensities
(10^3 cpm/g fr. w.)

Light intensity	5		100		1000		10,000	
Material	normal	albino	normal	albino	normal	albino	normal	albino
10^3 cpm/g	4600.0	5700.0	3100.0	9100.0	7400.0	11 200.0	52 000.0	8200.0
10^3 cpm/lux ...	...	...	31.0	91.0	7.4	11.2	5.2	0.8
a/n	1.2		3.0		1.5		0.2	

plants, when light intensity increased. The activity reaches its maximum at 1000 lux in albino leaves; it is somewhat lower at 10,000 lux illumination. By comparing the cpm/lux values one can see, that at 100 lux light intensity the ^{14}C activity fixed by the "solid" fraction of the albino leaves, is three

times as high, as the ^{14}C activity of the same fraction of the normal leaves. At 1000 lux this difference is smaller, but at this intensity the values are higher for albinos. At 10,000 lux — compared to normal leaves — the ^{14}C fixation by the "solid" fraction of the albino leaves decreases, and so the cpm/lux value remains appreciably below the values of the normal leaves. The a/n ratio shows a maximum at 100 lux, similarly to the corresponding values of Table 2, then it drops considerably at stronger illumination.

The decrease in protein content of the albino leaves during illumination may be explained by increased protein decomposition, as referred to also in the literature [50]. This view is supported also by experiments showing that the light sensitivity of the photosynthetic apparatus depended considerably upon protein synthesis [28, 30].

In our further investigations we have studied the distribution of ^{14}C incorporated into the various components of the alcohol soluble fraction. After exposition, 25 to 30 active spots appeared on the radiograms, made for the determination of the different components, and for the comparison of the amount of ^{14}C fixed by each of them. We succeeded in identifying 20 of these by selective developers, and R_f values: aspartic acid (1), glutamic acid (2), alanine (3), glycine + serine (4), phenylalanine (5), leucine (6), proline (7), tyrosine (8), glutamine (9), sucrose (10), phosphoglyceraldehyde (11), phosphoglyceric acid (12), malic acid (13), glycolic acid (14), succinic acid (15), citric-isocitric acid (16), α-ketoglutaric acid (17), glyceric acid (18), tartaric acid (19), fumaric acid (20).

The characteristic radiograms of the alcohol soluble components in the albino and normal leaves kept at 10,000 lux intensities, are shown by Fig. 1.

Fig. 1 shows clearly the relative difference in distribution of activities fixed by the alcohol soluble components of normal and albino leaves.

Aspartic acid, glutamic acid, alanine, glycine + serine show an intensive labelling in the leaves of normal plants. The organic acid content — compared to the albinos — is low at this light intensity. Chiefly malic acid, and among the amino acids aspartic and glutamic acid spots have strong activity in albino leaves.

On the basis of the qualitative evaluation of the radiograms, the components — characteristic of albinism and light effect — showing the greatest differences, were analyzed quantitatively. These components are the following: aspartic acid, glutamic acid, alanine, glycine + serine, malic acid, and glycolic acid. The active spots were dissolved, from the paper and their isotope content measured. The percentage distribution of the fixed ^{14}C per unit weight material is shown by Table 4.

It may be seen, from Table 4, that in the dark the activity is mainly due to glycolic acid, aspartic acid and malic acid. As light intensity is increased, the $^{14}CO_2$ content of amino acids increases in normal and decreases in albino

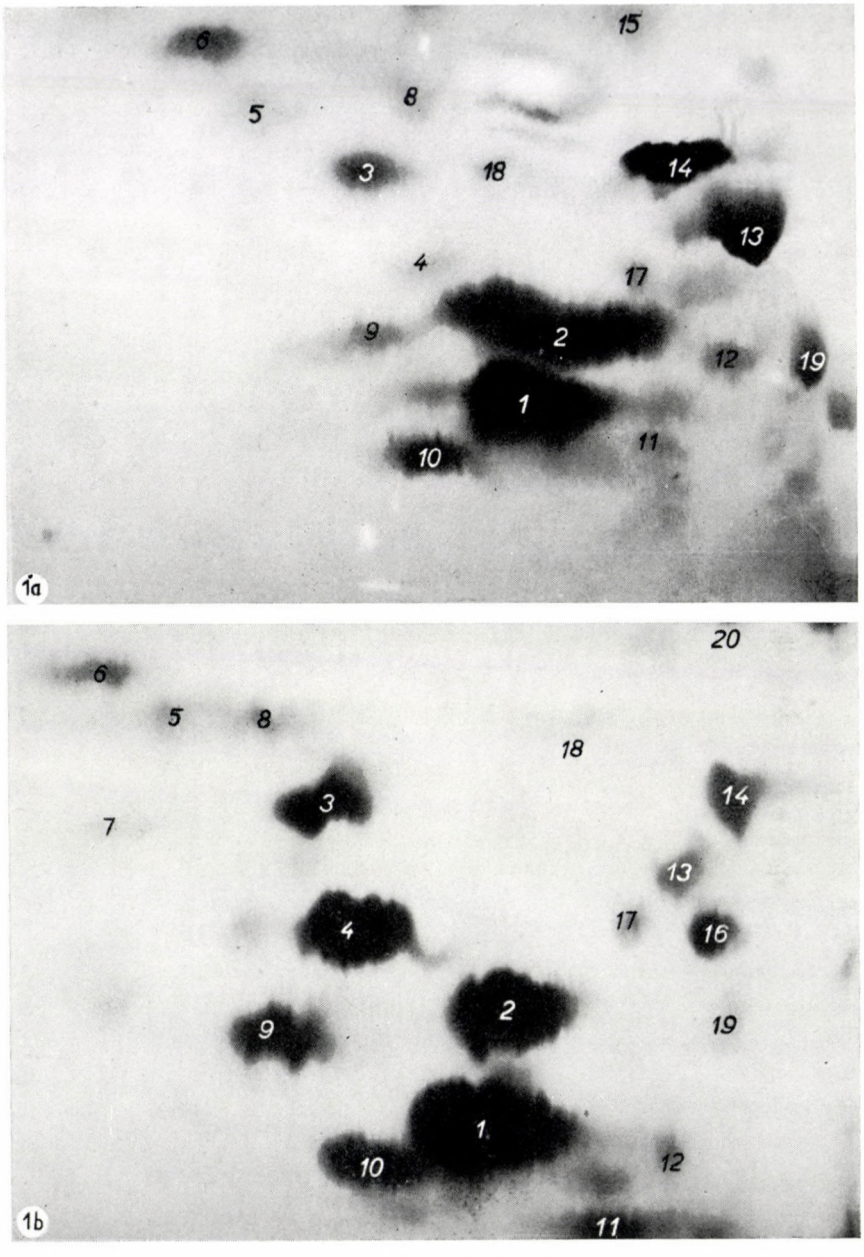

Fig. 1. Radiograms made from the alcohol soluble fractions of normal and albino leaves kept at 10,000 lux light intensity. *a* — albino, *b* — normal

leaves. The tendency is the same for glycolic and malic acids, but the differences between the normal and albino leaves are much smaller.

The a/n values obtained at different light intensities show that more ^{14}C is fixed, at 5 and 100 lux, by the alcohol soluble components of the albino leaves, than in the normal. The a/n values, belonging to these two light intensities, are nearly the same, only at 100 lux decrease in a/n concerning glycine + serine is observed. This shift in the ratio is due to the fact that the corresponding values of the albino drop to half of the 5 lux values. At 1000 lux the a/n ratio is low on account of the more intensive labelling of the alcohol soluble components in the normal leaves. Also, at this light intensity, the a/n value of glycine + serine is the lowest, simultaneously, the a/n value of glycolic

Table 4

The ^{14}C content of some of the alcohol soluble components of normal and albino leaves at different light intensities

Light intensity (lux)	5			100			1000			10,000		
Material	normal	albino	a/n	normal	albino	a/n	normal	albino	a/n	normal	albino	a/n
aspartic acid	4.0	6.0	1.5	2.4	4.0	1.7	45.5	4.8	0.16	2840	3.4	0.001
glutamic acid	2.2	2.5	1.1	2.1	2.4	1.2	20.1	2.8	0.14	735	3.6	0.005
alanine	0.8	1.2	1.5	0.6	0.9	1.5	5.0	0.8	0.16	226	0.8	0.004
glycine + serine ..	0.4	0.8	2.0	0.4	0.4	1.0	4.4	4.4	0.09	516	0.3	0.006
glycolic acid	5.0	7.4	1.5	4.5	6.2	1.4	16.4	5.2	0.32	58	3.7	0.06
malic-acid	2.7	3.5	1.3	2.1	3.2	1.5	6.2	3.4	0.55	35	2.9	0.08

and malic acid is markedly high. The situation is similar at 10,000 lux, the differences are, however, even greater: the a/n ratio of glycine + serine is the smallest, while the a/n value of glycolic and malic acid is appreciably higher than of the rest. At this light intensity the a/n ratio decrease for to aspartic acid is also more important.

The diagrams in Fig. 2 show ^{14}C fixation by the components at different light intensities. Light intensity is shown on the abscissae, while the ordinates epresent the perrcentage activity distribution related to the amounts (5×10^5 tpm) brought up.

The following values were obtained by quantitative evaluation of the components of the alcohol soluble fraction.

Aspartic acid : Aspartic acid ^{14}C activity of the normal leaves is lower at dim light than in the dark, while at more intensive light it significantly increases.

Activity decreases with increasing illumination in albino leaves. During $^{14}CO_2$ assimilation — in accordance with literature — aspartic acid is labelled both in dark and light [16, 35]. Fixation may take place directly, via oxalo-

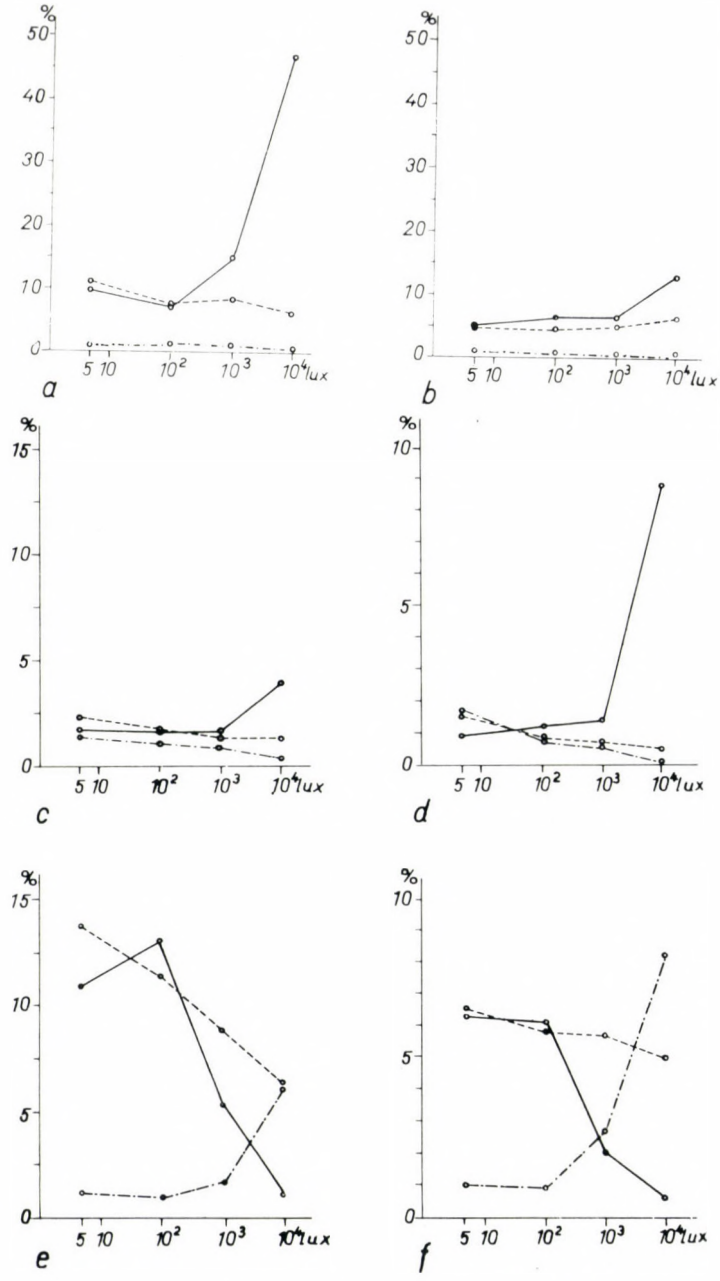

Fig. 2. The effect of various light intensities on the percentual distribution of the alcohol soluble components of normal and albino leaves. *a* — aspartic acid, *b* — glutamic acid, *c* — alanine, *d* — serine + glycine, *e* — glycolic acid, *f* — malic acid, normal: ————, albino: - - - - - -, a/n: - · - · - · - · -

acetic acid, from pyruvic acid of photosynthetic origin, or indirectly through the Krebs cycle [14, 15]. With stronger illumination — on account of more intensive protein synthesis — fixation into aspartic acid diminishes [2].

The possibility of these pathways may refer to the fact that the processes in normal and albino leaves are essentially different. Presumably dark fixation may be the only means of aspartic acid formation, because of the absence of photosynthetic $^{14}CO_2$ fixation in albino leaves at intensive illumination. This may be the pyruvic acid — oxaloacetic acid path.

Glutamic acid : Glutamic acid activity is nearly the same at low and medium light intensities in normal leaves, increasing only at 10,000 lux. This activity increase, however, is of much lesser extent than in the case of aspartic acid. The values were similar in albino leaves to those of the normal ones, at a lower level.

From the similar trend appearing in normal and albino leaves, the conclusion can be drawn, that the pathways of glutamic acid formation are the same. During ^{14}C assimilation, shortly after exposition, glutamic acid activity appears both in the light, and in the dark [5]. The amount of glutamic acid may be increased though at more intensive illumination by the increasing oxidation processes the inhibition of the Krebs cycle, however, interferes this process to a certain extent [40]. This would explain the relatively insignificant activity increase during illumination.

Some authors assume, that glutamic acid is formed from the reduction products of CO_2 (acetate, glycolic acid, phosphoenolpyruvic acid, alanine) [51]. This hypothesis would account for the fact that the glutamic acid content is lower in albino, than in normal leaves.

Alanine : ^{14}C activity in alanine is relatively low at less intensive illumination in normal leaves, while at high intensities (10 000 lux) it increases to a greater extent. As a contrast, in albino leaves the labelling of alanine is low at any light intensity.

Alanine is one of the characteristic products of photosynthesis [57]. The views are different as far as its formation is concerned. Earlier experiments made with $^{14}CO_2$ point out phosphoglyceric acid as its starting material [55]; according to recent data it forms by the reductive amination of phosphoenolpyruvic acid [35, 42]. The latter may explain the opposite activity change found in the albino leaves. So far, however, we were unable to interpret the high activity observed at 5 lux in normal leaves.

Serine + glycine : The change in the ^{14}C content of these two amino acids is similar to that of alanine, which is understandable, since during photosynthesis, starting from pyruvic acid, they form part of a common transformation process [44].

Glycolic acid : We have found that the activity of glycolic acid decreased appreciably at increasing light intensity in normal leaves. There is a decrease

also in albino leaves, but to a smaller extent. Several authors have shown [7, 8] that glycolic acid appears among the first products of photosynthesis in algae and in higher plants, and it would serve as intermediary towards the further — mainly amino acid — synthesis [54]. In our experiments, however, the considerable decrease of activity, upon increased light intensity, refers rather to connections with the increasing oxidation processes.

Malic acid : The ^{14}C activity of malic acid decrease considerably with increasing illumination in normal leaves. At the same time, the high activity of malic acid is not affected by the light intensity change in albino leaves.

There are two ways of formation of malic acid: one of them by ribulose-diphosphate carboxylase, the other by phosphoenolpyruvic acid carboxylase [38, 12]. LYTTLETON [37] and FULLER [26] were unable to find ribulose-diphosphate carboxylase in albino brome grass and xantha barley leaves, and so the Calvin cycle did not function, either. Their objects were plants without chlorophyll. It seems probable that dark fixation is the main pathway of malic acid formation also in the leaves of albino corn used in our experiments. This would explain why malic acid activity is not affected by different light intensities. At low light intensity there is a possibility for photosynthetic CO_2 fixation, by the leaves containing a considerable amount of pigment. Upon the effect of high light intensity, the $^{14}CO_2$ fixation is shifting toward the carbohydrates [41, 3], or rather, the malic acid formed in light is rapidly oxidized [38]. This problem could be solved with certainty by the examination of the labelling of the C-1 and C-4 atoms of malic acid.

When interpreting results, obtained in our experiments and found in literature, the question arises: how are the differences observed in the $^{14}CO_2$ assimilation of normal and albino plants related to albinism? It has been established that the differences observed in amino acid content show a parallelism relatively easy to trace with the changes in the pigment content [24, 23, 19]. According to certain data in literature, there are more free amino acids in the leaves of pigment defective mutants, than in normal leaves kept under similar conditions [25, 29]; at the same time, their protein content decreases [53]. This may be due to the destruction processes of the chloroplast structure [1]. The CO_2 photosynthetic fixation curve considerably flattens, on account of protein and pigment destruction processes due to high light intensity. It seems very probable, that the abnormal photosynthetic CO_2 fixation by the albino leaves is caused by troubled protein — pigment synthesis. From the diversity of the differences, however, it may be concluded that there are abnormalities at several points of metabolism.

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EINBAU VON $C^{14}O_2$ IN NORMALE UND ALBINO MAISBLÄTTER BEI VERSCHIEDENEN LICHTINTENSITÄTEN

Die $C^{14}O_2$-Assimilation von normalen und albino Maiskeimpflanzen wurde bei verschiedenen Lichtintensitäten verglichen und hierbei festgestellt, dass bei 5 und 100 Lux in die lipoidlösliche, alkohollösliche und unlösliche Fraktion der Albinoblätter mehr C^{14} eingebaut wird, als in die entsprechenden Fraktionen der normalen Blätter. Bei höherer Lichtintensität (1000, 10 000 Lux) wurde dagegen die Beobachtung gemacht, dass die obigen Fraktionen der normalen Blätter viel stärker markiert werden, als die der Albinoblätter, deren C^{14}-Gehalt bei diesen Lichtintensitäten schon eine abnehmende Tendenz aufweist. Die Albinoblätter zeigen zwischen 100 und 1000 Lux ein Maximum des C^{14}-Einbaues was auch durch die paralelle Erhöhung und Destruktion des Pigmentgehaltes veranschaulicht wird. Die Untersuchung der quantitativen Verteilung von C^{14} zwischen den einzelnen Verbindungen der alkohollöslichen Fraktion hatte gezeigt, dass das Licht ausser dem Ausmass der C^{14}- Inkorporation auch den Weg der Weitergabe stark beeinflusst. In den Blättern steigt der C^{14}-Gehalt an Alanin, Serin, Glycin und Asparaginsäure mit zunehmender Belichtungsintensität an, in den Albinoblättern wird dagegen über 100 Lux die C^{14}-Inkorporation in der Richtung der organischen Säuren verschoben. Aus den Versuchsangaben geht hervor, dass der Unterschied der Lichtintensität der normalen bzw. albino Blätter auch auf den Mechanismus der $C^{14}O_2$-Fixierung und der Weitergabe des assimilierten Kohlenstoffes auswirkt.

УСВОЕНИЕ $^{14}CO_2$ В ЛИСТЬЯМИ НОРМАЛЬНОЙ И АЛЬБИНОСНОЙ КУКУРУЗЫ ПРИ РАЗЛИЧНОЙ ИНТЕНСИВНОСТИ СВЕТА

Авторы провели сравнение ассимиляции $^{14}CO_2$ нормальных и альбиносных проростков кукурузы при различной интенсивности света. Установлено, что в липоидрастворимую, спирторастворимую и нерастворимую фракцию альбиносных листьев при освещении в 5 и 100 люксов включается больше ^{14}C, чем в соответствующие фракции нормальных листьев. Однако, при более сильной освещении (1000, 10 000 люксов) фракции нормальных листьев становятся гораздо сильнее мечеными, чем соответствующие фракции альбиносных листьев, содержание ^{14}C которых при этих интенсивностях света уже несколько снижается. Максимум усвоения ^{14}C в альбиносных листьях обнаруживается между 100 и 1000 люксами, что хорошо видно также из параллелного увеличения и деструкции содержания пигментов. Из количественного распределения ^{14}C между отдельными соединениями спирторастворимой фракции видно, что кроме степени включения ^{14}C свет в значительной степени влияет также и на его дальнейшую судьбу. По мере увеличения интенсивности освещения в нормальных листьях увеличивается содержание ^{14}C аланина, серина, глицина и аспарагиновой кислоты. Напротив этому, в альбиносных листьях выше 100 люксов усвоение ^{14}C сдвигается в сторону органических кислот. Полученные данные показывают, что разница между светочувствительностью нормальных и альбиносных листьев оказывает действие на механизм усвоения и последующей передачи $^{14}CO_2$.

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ON THE PHYSIOLOGICAL CONTROL OF CONNECTIVE TISSUE MAST CELLS

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Synopsis

The effect of cortisone, desoxycorticosterone acetate (DOCA), heparin, protamine sulfate and hyaluronidase on mast cells was studied in rats. Cortisone was found to influence the formation and destruction of mast cells. DOCA antagonizes the effect of cortisone only to a limited extent. Heparin given alone has no effect on the number and morphology of mast cells but administered together with cortisone it has such effect. These results suggest that cortisone may have a role in physiological control of mast cells.

Introduction

Mast cells represent characteristic cell types of the loose connective tissue and contain heparin, histamine and serotonine in their cytoplasm [3, 15]. Their most conspicuous feature is a metachromatic staining obtained after treatment with basic dyes. This gamma metachromasia is characteristic of heparin. The mast cells are apt to desintegrate with a release of stored heparin. As most mast cells are pericapillary localized the heparin released most probably enters the blood stream and leads to an increased level of serum acid mucopolysaccharides. The origin of the mast cells is not fully understood. Indeed, all cells carrying the characteristic metachromatic granules are termed as mast cells [4, 9]. It is well known from literature [18, 19] that fibroblasts or macrophages may transform into such cells by heparin uptake. Our earlier studies [6, 7, 8, 11] revealed an intense primary mast cell formation in rat thymus. Large and medium sized thymocytes take up the PAS positive substance of Hassall's bodies and cysts and transform it intracellularly into metachromatic substance, i.e. heparin. Cortisone plays an important role in the mast cell formation thus occurring in thymus [10, 12] by eliciting mast cell formation — parallel to the evacuation of thymus — and subsequent destruction of hypergranulated mast cells which leads to increased acid mucopolysaccharide level in blood serum.

There are several reports on the influence of cortisone on mast cells. A decrease of their number upon hormonal effect was observed by ASBOE-HANSEN [2] and CAVALLERO and BRACCINI [5]. Others, e.g. ARVY [1] in contrast found an increase of mast cell number after cortisone treatment.

Others again e.g. SMITH and LEWIS [17] and DEVITT, PIROZYNSKY and SAMUELS [13] regard the cortisone to have no effect on mast cell number and in general on these cells.

As in our experiments [10, 12] cortisone unequivocally produced the above described changes, in this study the effect of gluco- and mineralocorticoids on mast cell formation was tested also in loose connective tissue. For the sake of comparison some other substances having an affinity to heparinoids were also tested.

Materials and methods

The method of HIGGINBOTHAM [14] seemed to be most suitable to follow up the faee of mast cells in loose connective tissues. Approximately 10 ml air is inflated with a syringt into the subcutaneous connective tissue in the interscapulary area of rats. (The pouch obtained in this way is similar to the air pouch which is the first stage in the preparation of SELYE's granuloma pouch [16].) The skin is dissected to leave only a two-three layer thick connective tissue wall above the pouch. The air is then aspirated and replaced by physiological saline. The substances to be tested could be introduced at will into the fluid-filled pouch.

HIGGINBOTHAM removed and placed the membrane on a slide prior to staining. In our modification 1 per cent toluidine blue in physiological saline was dropped on the membrane in vivo which resulted in a red staining of mast cell granules. Excess stain was rinsed with saline. The number and/or the numerical and morphological changes of mast cells due to different agents were ascertained under a stereomicroscope in vivo. Changes were expressed as per cent of the initial number observed in the same field. If certain substances were repeatedly administered after washing of the pouch or were applied after previous treatment with other substances, the changes were expressed as per cent of the number observed immediately before the last treatment.

The experiments were performed on a total of 60 Wistar rats of about 150 g weight. Anaesthesia was performed with intraperitoneally administered Intranarcon. The observations lasted 15 minutes. After this time the wall of the pouch was removed, placed on a slide, fixed with Carnoy and stained with toluidine blue. Microphotographs presented in this paper were made of such membranes.

The effect of following substances was tested: Prednisolon (Di-adreson F-aquosum Organon) — 5 mg; desoxycorticosterone acetate (Docaquosum, Organon) — 2 mg; heparin (Heparin pulvis, 100 IU/mg Richter) — 4 mg; Protamine sulphate (Roche) — 5 mg; hyaluronidase (Hyason, Organon) — 50 IU.

Results and discussion

Table 1 shows the results of observations for 3 and 15 min. (i.e. mast cell numbers as per cent of the numbers observed before the administration of the substances tested). These moments were chosen as preliminary observations revealed that increase of the mast cell number attains maximum value after 3 minutes and that no further destruction is observed after 15 minutes with the exception of protamine sulphate which elicited maximal destruction as early as after 5 minutes.

The 1 per cent solution of toluidine blue produced a sharp staining of the mast cells but had no adverse effect on the cells within the duration of the experiments. This is shown by the fact that not only destruction but also

Table 1

Changes of mast cell number
(% ± S. D. of original value)

Treatment	Effect after	
	3 min.*	15 min.**
Heparin	no effect	no effect
Cortisone	11% ± 6.5% ($p < 5$)	—7% ± 6.5% ($p < 1$)
Cortisone and heparin	30% ± 11.0% ($p < 1$)	—25% ± 8.0% ($p < 0.1$)
Repeated administration of cortisone and heparin	15% ± 4.0% ($p > 5$)	1% ± 4.0% ($p > 5$)
DOCA	no effect	no effect
DOCA and heparin	no effects	no effects
Cortisone, DOCA and heparin	10% ± 3.0% ($p > 5$)	—4% ± 9.0% ($p > 5$)
Protamine sulphate (5 min.)	—61% ± 9.0% ($p < 0.1$)	
Cortisone after protamine sulphate	no further effect	no further effect
Hyaluronidase	no effect	no effect

* Significance against initial value
** Significance against 3 min. value

increase of mast cell number occurred further by the fact that the cells regained its ability to stove after washing and removal of the pouch content.

The experiments revealed that cortisone has a marked effect on mast cells resulting in changes both of their number and of their morphology. Cortisone produced a 10 per cent increase of cell number after 3 minutes followed by a destruction of the hypergranulated cells amounting to a 10 per cent decrease of the number after 15 minutes. The simultaneous administration of cortisone and heparin into the pouch gave a more explicit effect. A 30 per cent increase and a subsequent 25 per cent decrease was observed after combined treatment. Heparin alone has no effect on mast cell number. These results suggest the assumption of two factors controlling the mast cell number: (1) cortisone which elicits mast cell formation and (2) heparin which must be present to enable the transformation of cells into mast cells. Heparin normally present in the circulating blood can presumably also be taken up by the cells — this might account for the increase of mast cell number after cortisone administration in itself — but excess heparin is promoting the transformation. Cortisone produces increased granulation in addition to higher cell number (Figs 1 and 2). This hypergranulation is presumably one of the

main causes of cell destruction and consequent decrease of mast cell number (Figs 2 to 4). In the presence of excess heparin, cortisone induces not only increased mast cell number but, subsequently increased destruction as compared with the effect of cortisone alone. These observations support the above assumption.

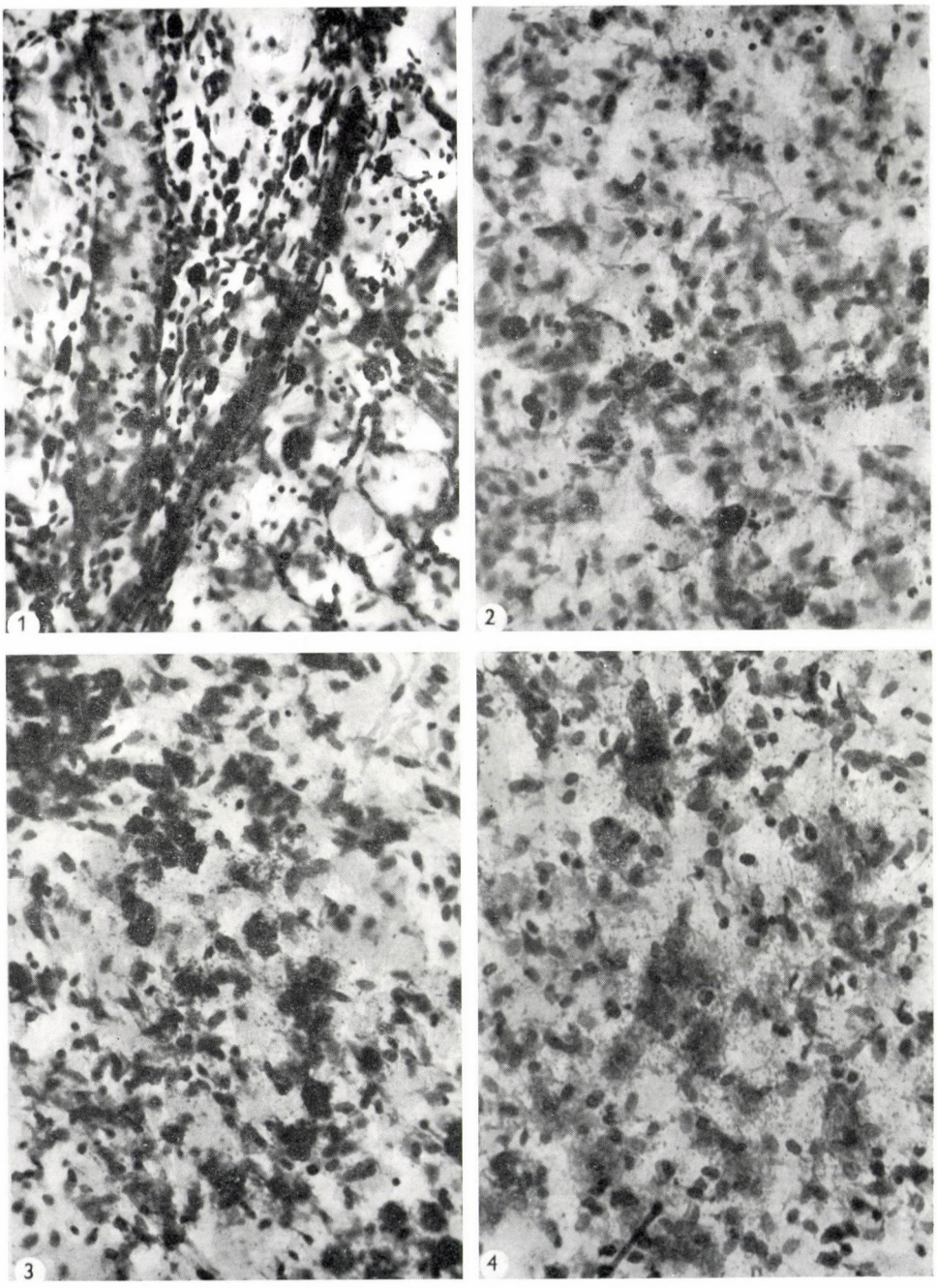
The prevalence of increase of mast cell number at the beginning of cortisone effect and the subsequently appearing decrease might explain the inconsistencies in the findings of other authors. Those who made only short range experiments found increased cell number whereas those counting only after a while found decreased number. Similar explanation has most probably given for observations revealing no changes in cell number. Changes of cell number are, however, most probably also dose dependent in addition to the time factor.

Repeatedly administered cortisone has a similar but less pronounced effect. If the pouch is washed after short cortisone treatment and the treatment is repeated after a certain time, again an initial increase followed by a decrease of mast cell number was observed. It can not be decided, however, whether heparin was taken up by the same cells or by other cells indifferent so far.

The origin of the cells transforming into mast cells under cortisone effect presents an interesting problem. SMITH and LEWIS [18] produced a destruction of peritoneal mast cells by intraperitoneal injection of water. The released granula were taken up by fibroblasts, macrophages and leucocytes which then gave "abnormal mast cells". Others, e.g. VELICAN and VELICAN [19] observed the transformation of macrophages into mast cells which were, however, not completely identical with genuine mast cells. No differences between newly formed and preexistent mast cells could be observed in our experiments which allows the assumption that the more or less indifferent cells giving rise to new mast cells are potential mast cells even if they do not contain actually metachromatic substances. The existence of such potential mast cells was already assumed in an earlier paper [9].

Desoxycorticosterone acetate, a mineralocorticoid, alone or given with heparin had no effect on the number or morphology of mast cells but slightly blocked the increase of cell number and more markedly the destruction of mast cells under cortisone effect if given simultaneously with cortisone and heparin. This result corresponds to earlier findings of increased heparin storage in thymus under the effect of DOCA [12].

The effect of protamine sulphate should be especially mentioned. This strongly basic protein has great affinity to the highly negative heparin and this is most probably the explanation of its decreasing effect on mast cell number. The binding is so intense that even cortisone can not induce the cells to take up more heparin. Presumably the heparin and protamine sulphate form a complex which is inaccessible for the cells or the protamine sulphate kills the



Figs 1 to *4*. Wall of the pouch stained with toluidine blue. *Fig. 1*. Normal mast cells. *Fig. 2*. Commencing destruction of hypergranulated mast cells under cortisone effect. *Fig. 3*. Increased destruction of mast cells. *Fig. 4*. Very intense destruction producing only metachromatic patches where no cell boundaries are seen. ×200

cells and no substrate remains for cortisone effect. Both suggested modes of action show the effect of protamine sulphate to be non-physiological.

Hyaluronidase had no effect on mast cells, showing that the mucopolysaccharide present in these cells is no hyaluronic acid-like substance but highly complex heparin. This finding renders improbably that mast cells were responsible for the production of connective tissue ground substance [3, 15].

Of all substances tested only cortisone produced a regular biphasic effect. This finding and earlier results concerning mast cell formation suggest that cortisone is the physiological regulator substance of mast cell formation and destruction. This is to say that this substance occurs in normal organism and is not artificially introduced as protamine sulphate, histamine liberators, etc. Cortisone has central (on the thymus) and peripheric (on connective tissue) effect. Only further studies may, however, show whether other substances, not yet tested, have an effect on mast cells.

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DIE PHYSIOLOGISCHE REGULATION DER MASTZELLEN DES BINDEGEWEBES

Es wurde die Wirkung von Cortison, Desoxycorticosteronazetat (DOCA), Heparin, Protaminsulphat und Hyaluronidase auf Mastzellen untersucht. Cortison beeinflusste im Versuch die Bildung und den Zerfall von Mastzellen. DOCA wirkte nur in geringem Masse antagonistisch zum Cortisoneffect. Allein verabreicht erwies sich Heparin als unwirksam in bezug auf Morphologie und Anzahl der Mastzellen, war aber mit Cortison wirksam. Nach den Ergebnissen spielt Cortison bei der physiologischen Regulation der Mastzellen eine gewisse Rolle.

ФИЗИОЛОГИЧЕСКИЙ КОНТРОЛЬ ТУЧНЫХ КЛЕТОК СОЕДИНИТЕЛЬНОЙ ТКАНИ

Авторы исследовали влияние кортизона, ацетата дезоксикортикостерона (ДОКА), гепарина, протамин-сульфата и гиалуронидазы на тучные клетки. Кортизон оказал влияние на образование и распад тучных клеток. Один только ДОКА в малой степени препятствовал влиянию кортизона. Один только гепарин не оказал влияние на число и морфологию тучных клеток, однако применяя вместе кортизоном оказал заметное влияние. Исходя из полученных результатов можно сказать, что кортизон играет роль в физиологическом контроле тучных клеток.

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SUGAR ANTAGONISMS IN PLANT TUMOUR CELLS INDUCED BY 2,4-DICHLOROPHENOXYACETIC ACID

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Synopsis

The effects of different sugars on the tumorous growth of potato tissue cultures have been compared. It was found that the 12 different sugars, added to the medium, may be classified into three types depending on their effect exerted on the tumorous growth of potato tissue cultures: (a) sugars with a positive stimulating effect on growth of cultures (sucrose, trehalose, D-glucose); (b) sugars ineffective or poorly utilized (glycerine, D-ribose, D-fructose, maltose); (c) definitely toxic, antagonizing even the utilization of sugars ensuring basic growth (L-arabinose, D-galactose, L-sorbose, lactose). Comparing the effect of different sugars upon tissue cultures with the data found in literature, the conclusion was drawn that the observed antagonisms probably are due to competition connected with epimerization difficulties.

Introduction

Complicated stimulating and inhibiting effects, depending on the object and sort of sugar have been observed while studying the influence of different sugars on the growth of plant tissue [14, 19, 27, 30]. Certain sugars (galactose, lactose, arabinose) in root tips have shown a definite toxic effect: they inhibited even the metabolism of sugars originally present in plant tissues [13, 16, 29].

It is well known that sugar metabolism is changed by the auxonherbicide 2,4-D: invertase activity is increased [25]; respiration tends towards direct oxidation [21]; several ketonic acids are mobilized by the deamination of amino acids [8]. It was shown that 2,4-D, as a growth regulator decreases sugar incorporation into pectin and cellulose molecules, which indirectly contributes to the continuation of growth in an undifferentiated manner [28].

The object of the present survey was to study the influence of different sugars on tumorous growth characteristic of the auxin effect of 2,4-D on potato tissue cultures.

Material and methods

The tissue cultures serving as experimental material were prepared from the tubers of the 2,4-D sensitive "Gül Baba" (GB), and the relatively resistant "Margit" (M) varieties (10). The material was obtained from the National Institute of Agricultural Botanics (Tápiószele) in 1959, and was grown at the Alsógöd Biological Station of this University.

50 to 100 g tubers were used for the experiments. The surface of the tubers were washed according to surgical sterility, and sterilized for 20 minutes in a 2% solution of sodium-benzenesulphonechloramide. Pieces of tubers of optimum size and weight for growth [11] (6 mm × 6 mm × 0.5 mm and 25 ± 1.6 mg) were cut from the tubers, under sterile conditions. The cultures were grown on [7] White's modified nutrient solution [24] containing 10^{-4} M2,4-D, 0.05% caseinhydrolysate and 0.058 M different sugars equivalent to 2% sucrose.

The effects of the different sugars were studied in three series, each comprising 80 to 120 pieces of tissue. Tissue culture growth was evaluated on the basis of fresh weight gain after 14 days.

Results

Experiments were concerned with glycerine, pentoses, hexoses, and disaccharides generally occurring in plants.

Table 1 shows the variation of fresh weight in "Gül Baba" liable to intensive tumour formation as affected by 2,4-D and the less intensively growing "Margit" cultures, grown on culture media containing different sugars.

Table 1

The growth of potato tissue cultures on nutrient mediums containing different sugars and 10^{-4} M 2.4-dichlorophenoxyacetic acid

Sugars	Gül Baba			Margit		
	mg ± s	weight gain		mg ± s	weight gain	
		rate	%		rate	%
Base line growth without sugars added	83 ± 20.6	3.3	100	48 ± 12.6	1.9	100
Triose						
Glycerine	101 ± 36.3	4.1	124	43 ± 12.9	1.7	89
Pentoses						
D-ribose	75 ± 20.4	3.0	91	34 ± 1.7	1.4	73
D-xylose	37 ± 4.6	1.5	46	30 ± 2.0	1.2	63
L-arabinose	48 ± 17.3	1.9	58	41 ± 6.0	1.6	84
Hexoses						
D-glucose	119 ± 27.5	4.6	139	75 ± 20.4	3.0	158
D-fructose	97 ± 44.7	3.9	118	49 ± 8.5	2.0	110
D-galactose	28 ± 1.4	1.1	33	29 ± 2.2	1.2	63
L-sorbose	28 ± 1.0	1.1	33	29 ± 1.7	1.2	63
Disaccharides						
Sucrose	122 ± 32.5	4.9	149	87 ± 25.4	3.5	184
Trehalose	106 ± 21.1	4.3	130	103 ± 31.9	4.1	216
Maltose	75 ± 34.2	3.0	91	35 ± 9.7	1.4	74
Lactose	28 ± 1.0	1.1	85	29 ± 1.4	1.2	63

The rate of weight gain is related to the initial 25 mg fresh weight

From Table 1 it may be seen that tissue cultures significantly increase also on sugar free medium. Growth takes place by the utilization of the inner reserve: the degrading starch [6]. This basic growth (100) conspicuously shows the sensitivity of the two species as far as 2,4-D is concerned: "Gül Baba" increase its weight threefold during the growing period, while "Margit" only doubles.

Table 2

The interaction of different sugars in the growth of Gül Baba tissue cultures

No	Sugars	mg ± s	weight gain	
			rate	%
1	Basic growth without sugar	81 ± 18.0	3.2	100
2	1% sucrose	104 ± 30.6	4.2	128
3	,, ,, + 1% D-ribose	106 ± 25.0	4.2	131
4	,, ,, + 1% D-xylose	88 ± 21.0	3.5	109
5	,, ,, + 1% L-arabinose	92 ± 18.5	3.7	113
6	,, ,, + 1% D-glucose	108 ± 24.0	4.3	133
7	,, ,, + 1% D-fructose	93 ± 26.4	3.7	114
8	,, ,, + 1% D-galactose	30 ± 3.6	1.2	37
9	,, ,, + 1% L-sorbose	27 ± 5.3	1.1	33
10	,, ,, + 1% sucrose	107 ± 18.5	4.2	132
11	,, ,, + 1% lactose	46 ± 28.1	1.8	57

Glycerine has a slight stimulating effect on the growth of GB cultures while it has no effect upon M. As to pentoses, D-ribose is not utilized by GB, but it inhibits the growth of M. Both varieties result in a lower growth rate as compared with basic growth in media containing D-xylose and L-arabinose.

From hexoses, D-glycose is utilized very efficiently by the tissue cultures. Basic growth was not effected in either variety by D-fructose. D-galactose and L-sorbose proved to be toxic: weight increase was practically inhibited.

Among the disaccharides applied sucrose seemed to be optimal for GB, and very beneficial for M. In GB trehalose very closely approached and in M surpassed the growth promoting effect of sucrose. Maltose proved to be indifferent in both varieties. When feeding lactose a toxic effect, similar to that caused by D-galactose was observed.

To study the mode of action of the different stimulating and inhibiting sugars, their effect on the growth of tissue cultures was examined in the presence of sucrose. Experimental results are condensed in Table 2.

Fig. 1 shows the characteristic picture of the tissue cultures obtained in experimental variants presented in Table 2.

From Table 2, and Fig. 1 it appears that D-ribose does not influence the utilization of sucrose while D-xylose and L-arabinose even if applied together with sucrose cause a weight gain surpassing the basic growth only to a small extent. For sucrose utilization D-glucose is indifferent; D-fructose has a slight inhibiting effect. D-galactose and L-sorbose in the presence of

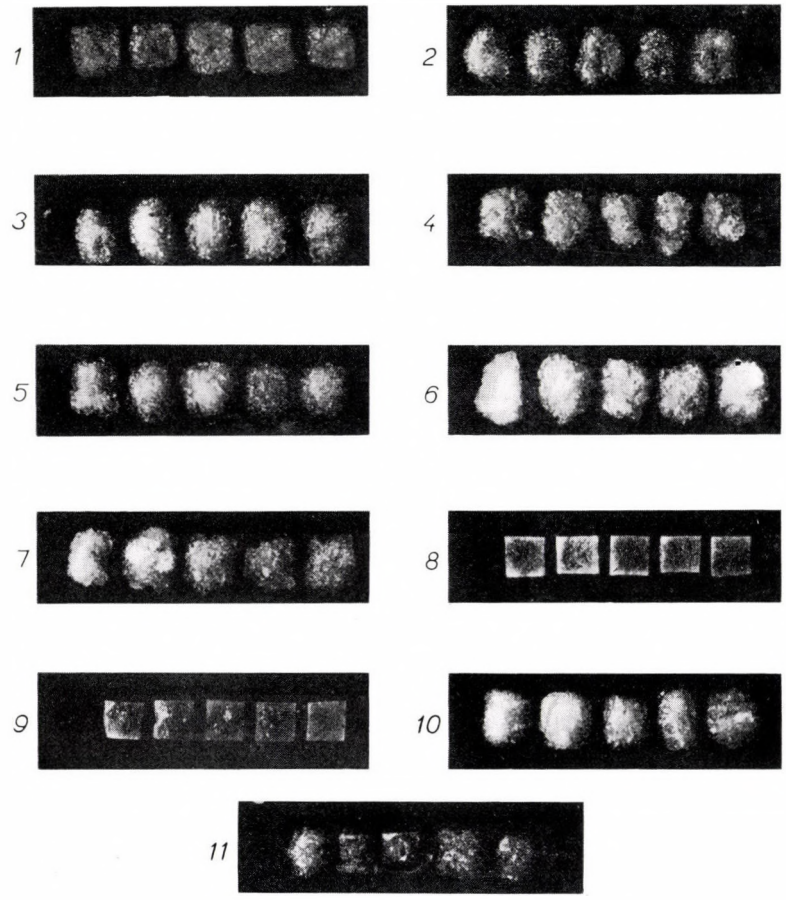

Fig. 1. The growth of potato tissue culture in growth media containing different sugars and sucrose (10^{-4} M 2.4-D; the concentration of sugars was 1% + 1%). Serial numbers the same as in Table 2

sucrose are similarly toxic as when applied separately. The toxic effect of lactose on the other hand is partly balanced by sucrose. However, it should be noted that the cultures from the individual potato tubers widely differred as far as the lactose inhibiting effect of sucrose was concerned, which was reflected by the unusually high standard deviation.

Discussion

The 12 sugars used may be classified into three types based on the effect exerted on the growth rate of tumorous potato tissues:

a) Definite growth promoters (sucrose, trehalose, D-glucose);

b) Ineffective or poorly utilized (glycerine, D-ribose, D-fructose, maltose);

c) Explicitly toxic i.e. antagonizing even the utilization of sugars assuring basic growth (L-arabinose, D-galactose, L-sorbose, lactose).

The composition of these groups indicates that the effect produced by sugars on potato tissue cultures does not depend upon the number of C atoms of the sugars.

Among the stimulating sugars sucrose serves as a standard energy source in plant tissue cultures [32]. According to our knowledge concerning its mode of utilization, sucrose absorption is uneffected in the presence of 2,4-D [4, 12]. In metabolism, UDP serves as carrier of glycosyl radical [23]. Since PO_4 uptake [3, 9] and phosphorylase activity are reduced by 2,4-D [33], sucrose utilization through fermentation is suppressed, and it is metabolized mainly through the pentose cycle [2, 21].

The effect of trehalose is similar of that of sucrose. This may be correlated with glycose, involved in the structure of both and with their C-1-α glycosidic bond. The growth promoting action of trehalose is particularly conspicuous in M cultures. Further investigations are needed to explain this phenomenon.

D-glycose resembles the previously mentioned sugars in the mode and extent of its action upon growth. In cases, when the effect of sugars is indifferent or only slightly stimulating tumorous growth of potato tissue cultures, permeability or utilization troubles may be involved.

In connection with glycerine it was shown [15] that tissue cultures prepared from different plant species utilized glycerine to various extent, and sometimes only after several passages. NICKEL and BURKHOLDER [27] succeeded to grow viral plant tumour on glycerine as a carbon source; the same was achieved by HILDEBRANDT [19] using the normal tissues of several species. GB cultures utilized glycerine appreciably in our experiments, with M cultures, however, this was not observable.

There are no data available in literature concerning the way of utilization of D-ribose in higher plants. The catabolism of D-ribose in bacteria occurs by phosphorylation [22]. It was not possible to stimulate the pentose cycle, from outside, by D-ribose in animal tissue and yeast [20]. Similar phenomenon was observed in potato tissue cultures, moreover the development of M cultures was slightly inhibited by D-ribose. It was possible to lift the inhibition by sucrose.

According to literature D-fructose is utilized similarly to glucose [19], our experiments, however, did not support this view: D-fructose was found to be considerably more poorly utilized.

Maltose did not appear to be a suitable carbon source for either variety. Similar results were obtained by ARYA and co-worker [1] in *Vitis* tissue cultures. The absence of the growth promoting effect in the case of maltose is presumably due to the fact that its glucose molecules, unlike to trehalose, form a C-4-β glucosidic bond. This view is supported by data in literature, giving account of the inefficiency of cellobiose containing C-4-α glucosidic bond [18].

It is characteristic of sugars having a positively toxic effect on potato tissue cultures, that they differred in the configuration of one of their C atoms, from more or less similar analogues.

The toxicity of D-xylose was observed also on the growth of root tips [13]. Pentose fermentation data on microorganisms may give an explanation for this [17]. It was shown that C-4 epimerase was needed to the interconversion of D-xylulose and D-ribulose, which possibly is missing from the tissues of the higher plants. In connection with the development of inhibition it must be supposed, however, that exogenic xylose may be interconnected with some branch of sugar metabolism, engaging there one of the major mediators, possibly UDP [26].

There may be a similar explanation for the toxic effect of L-arabinose. With the latter, however, C-4 is the critical C atom for epimerization. The competition of D-xylose and/or L-arabinose with the normal metabolites may not be very hard, because it is possible to suppress their growth inhibitory effect by adding sucrose.

The toxicity of D-galactose is explained by transport-antagonism [5]. Since the latter is not to be thought of in tissue cultures, it may be suggested the toxic effect of D-galactose is connected with C-4 configuration contrary to glucose and fructose. The steric difference forms an inhibition for the metabolic incorporation of galactose, if the tissue does not possess sufficient epimerase activity when C-4 makes a Walden turn. L-sorbose toxicity can be explained similarly when the necessity for epimerization arises between L-sorbose and D-fructose for the C-5 atom. D-galactose and L-sorbose have a stronger toxic effect, possibly involving metabolism at a different point than D-xylose and L-arabinose, since it is not possible to suppress the effect by 1% sucrose It was possible to successfully suppress galactose toxicity in root tips by ATP and inorganic PO_4 [13].

The toxic effect of lactose is probably attached to its D-galactose content. It is interesting to note that in the presence of sucrose this inhibition is partly removed which indicates that the attachment of the larger lactose molecule to the inhibitory point is less stable, than that of D-galactose.

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ZUCKERANTAGONISMEN IN DEN DURCH 2.4-DICHLORPHENOXYESSIGSÄURE INDUZIERTEN PFLANZLICHEN TUMORGEWEBEN

In Kartoffelgewebekulturen wurden die Wirkungen verschiedener Zucker auf das Tumorwachstum verglichen. Es wurde festgestellt, dass vom Gesichtspunkt der auf das Ausmass des Tumorwachstums ausgeübten Wirkung die 12 in das Nährmedium verabreichten Zucker in drei Typen eingereiht werden können: (a) das Wachstum der Gewebekultur ausdrücklich fördernde (Saccharose, Trehalose, D-Glucose); (b) wirkungslose oder nur wenig verwertbare (Glycerin, D-Ribose, D-Fructose, D-Maltose); (c) ausgesprochen toxische Zucker, welche sogar die Verwertung der das Grundwachstum fördernden Zucker antagonisieren (L-Arabinose, D-Galaktose, L-Sorbose, Lactose). Aus der vergleichenden Untersuchung der Wirkung verschiedener Zucker auf das Gewebewachstum und aus den Literaturangaben wurde gefolgert, dass die beobachteten Antagonismen wahrscheinlich mit einer Kompetition zu erklären sind, die mit Schwierigkeiten der Epimerisation verbunden ist.

АНТАГОНИЗМ САХАРОВ В ИНДУЦИРОВАННЫХ 2,4-ДИХЛОРФЕНОКСИУКСУСНОЙ КИСЛОТОЙ РАСТИТЕЛЬНЫХ ОПУХОЛЕВЫХ ТКАНЯХ

В культуре тканей картофеля авторы сравнивали влияние различных сахаров на рост опухолевых тканей. Установили, что с точки зрения влияния на стёпень роста опухолей тканевых культур картофеля, добавленные к питательному раствору 12 разновидностей сахаров можно распределить в три группы: а) сахара, определенно стимулирующие рост культуры (сахароза, трегалоза и D-глюкоза); б) сахара, недействительные, или слабо используемые (глицерин, D-рибоза, D-фруктоза, мальтоза); в) сахара, определенно токсичные, т. е. подавляют использование сахаров, обеспечивающих основной рост (L-арабиноза, D-галактоза, L-сорбоза, лактоза). Сопоставляя различное действие отдельных сахаров на рост тканей с литературными данными, авторы пришли к выводу, что обнаруженные антагонизмы по всей вероятности основываются на компетиции, связанной с эпимеризационными трудностями.

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A STUDY OF THE DIAMETER DISTRIBUTION OF THE SECRETORY LIPID VESICLES IN THE HARDERIAN GLAND

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Synopsis

The authors have studied the diameter distribution of the secretory elements of the Harderian gland, the lipid droplets or vesicles, using a statistical method. According to the observations made, significant differences in lipid vesicular diameters can be demonstrated in the native glandular homogenate smears, electron microscopic preparations and chromodacryorrhoeic secretions obtained from infantile and adult animals, in the sense that the granules are the smallest in the infantile animal's gland, larger in the adult animal's gland and largest in the chromodacryorrhoeic secretion.

Introduction

The Harderian gland is an intraorbital gland which occours in most land-vertebrates and contains a high amount of porphyrin in certain rodents. Light microscopy shows the cytoplasm of the epithelial cells of the gland of tubulo-alveolar structure to be filled with fine sudanophilic lipid granules; accordingly, in embedded sections the cytoplasm of the glandular acinar cells is of foamy structure. In the lumina of the acini and the efferent ducts brownish, presumably porphyrin, crystals are found. Electron microscopic examination reveals that the glandular epithelial cells are filled with innumerable vesicles, possessing well-defined membranes.

As to the function of the apocrine gland, the secretion of the gland appears as a fluid rich in lipids and may contain porphyrins, too. In the state of hyperfunction blood-like "tear drops", resulting from massive porphyrin secretion, appear in the palpebral fissure (chromodacryorrhoea) [7].

In agreement with the results of KANWAR [2], obtained by light microscopy, earlier investigations conducted in this Department indicated that the lipid vesicles building up the glandular epithelial cells had complex structure [4]. These observations supplied information about the genesis of the vesicles, maturation of the gland and development of the glandular epithelial cells. Further investigations [3] showed that in the Harderian gland of the rat the biosynthesis of porphyrin differred from that in the liver and the kidney, and was suggestive rather of the analogous process taking place in the bone marrow and in chloroma. The porphyrine content of the Harderian gland

increases with the advance of age [6], and correspondingly porphyrin biosynthesis is less definite in the glands of young animals than in those of older ones [1]. The observations concerning the Harderian gland made so far in this Department [3, 4, 5, 6] in their totality point to the fact that the number of lipid vesicles originating in the cytoplasm increases gradually with the maturation of the glandular epithelial cells, while the lipid vesicles fuse to form major structures and these appear in the secretion. The increase in vesicle count accompanying the maturation of the glandular epithelial cells seems to run parallel with the changes in the porphyrin content of the gland. Moreover, ORBÁN and KELÉNYI [5] reported that according to their electron microscopic observations concurrently with the maturation of the glandular epithelial cells not only the number, but also diameter or size of the vesicles showed a gradual increase.

In the immature glands of infantile animals a few lipid vesicles of small diameter can be found, while in the glandular acini of bigger animals they occur in masses and seem to have larger diameters.

The function of the Harderian gland seems to be correlated primarily with the lipid vesicles. Therefore it was expected that by analysing more exactly the differences in diameter of the vesicles by electron microscopy, a deeper insight might be gained into the process of glandular function. In these investigations the diameters of the lipid vesicles in the Harderian glands of animals of different ages were statistically analysed and the diameter distribution in glandular hyperfunction, or chromodacryorrhoea studied. The results confirm the suggestions made on the basis of the electron microscopic findings, since the diameters of the lipid vesicles appeared to be smaller in the young and larger in the adult animals.

Methods

Infantile and adult albino rats of 30 and 220 to 280 g b. w., respectively, kept under the same conditions, were used. The animals were decapitated under light ether anaesthesia, exsanguinated, the Harderian glands removed, weighed, minced and homogenized at a concentration of 100 mg wet tissue/0.5 ml saline in a Potter glass homogenizer with the same number of brayings (25×). The native smears of the fresh homogenates were covered with saline and examined by phase contrast microscopy. Setting the secondary magnification to exactly 2000×, the enlarged, positive pictures were evaluated by means of a TGZ-3 semi-automatic granule counter (IPTC, Zürich), in the form of distribution curves plotted according to exponential progression. The apparatus records the vesicles in 48 orders of magnitude.

In connection with the evaluation of the smears obtained from the glandular homogenates it was suggested that homogenation might cause fragmentation of the lipid vesicles. To make this clear, we homogenized manually and/or mechanically the glandular specimens in saline for different periods, and compared the granule distribution curves of the substances thus obtained with those of glandular specimens squashed with a cover-slip. The observations made it clear that the 25× homogenation in the Potter homogenizer produces no appreciable fragmentation of the vesicles. This is indicated also by the fact that the vesicle distribution curves obtained electron microscopically in embedded material had the same shape as those of the native homogenates.

We plotted the distribution curves also of the electron micrograms of ultrathin sections of OsO_4-fixed, ethanol-dehydrated and methacrylate-embedded Harderian glands, using the Elmi-D_2 Zeiss electron microscope of the Central Laboratory, Medical University of Pécs, and the BS 242 A type Tesla apparatus of the Electron Microscopic Laboratory, Coal Mining Trust of Pécs.*

Chromodacryorrhoea was induced by a single intravenous injection of 2 per cent pilocarpine or 20 per cent choline chloride, in 100 mg/kg doses. The smears of the excretion were covered, photographed and evaluated in the same way as described above.

Results

The Harderian glands of 5 animals of the 30 g weight group, and of 8 animals of the 220 to 280 g weight group were subjected to a study, measuring 1000 vesicle diameters in each case. The distribution curves obtained indicate

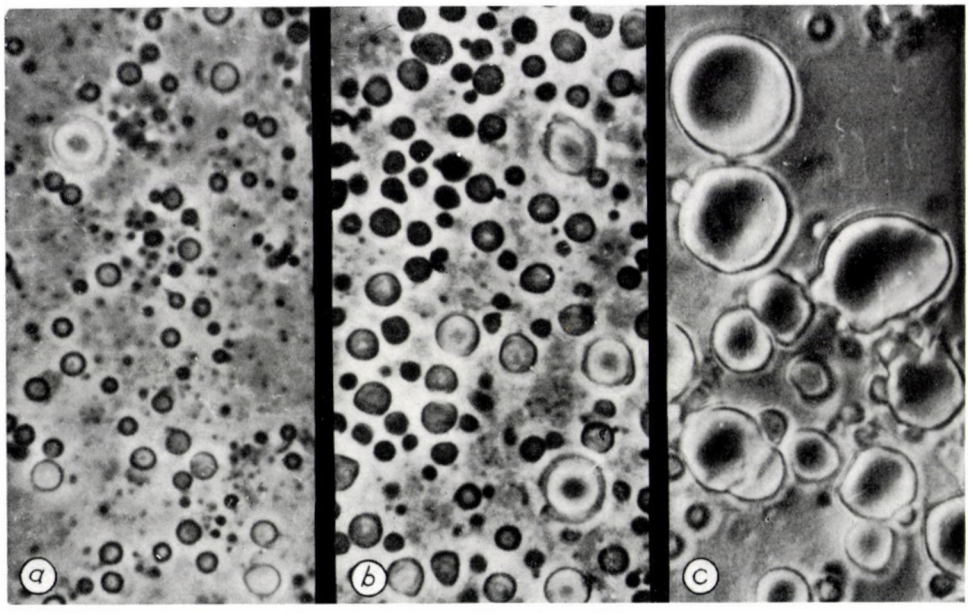

Fig. 1. Phase contrast micrograms of native smears of a) Harderian glands of infantile, b) adult rat and c) chromodacryorrhoeic excretion. × 2000

that with the infantile animals the maximum of the distribution of vesicle diameters fell into the 1.29 ± 0.07 μ order of magnitude, as compared with the 1.66 ± 0.18 μ value found in adults. The phenomenon indicates that the majority of the vesicles of the adult, actively functioning glands are larger than those of the almost inactive, immature, hardly secreting ones. The dif-

* We wish to express our thanks to the Coal Mining Trust of Pécs, and to István Gyurkó, chemical engineer, in particular, for having placed the apparatus at our disposal.

ference in the maxima of the vesicle diameters between the infantile and adult animals is significant ($p < 0.001$).

Fig. 1 shows the native smears of the glandular homogenates of infantile and adult animals, as well as that of the native smear of the chromodacryorrhoeic discharge.

Fig. 2 shows the vesicle diameter distribution curves of infantile and adult animals.

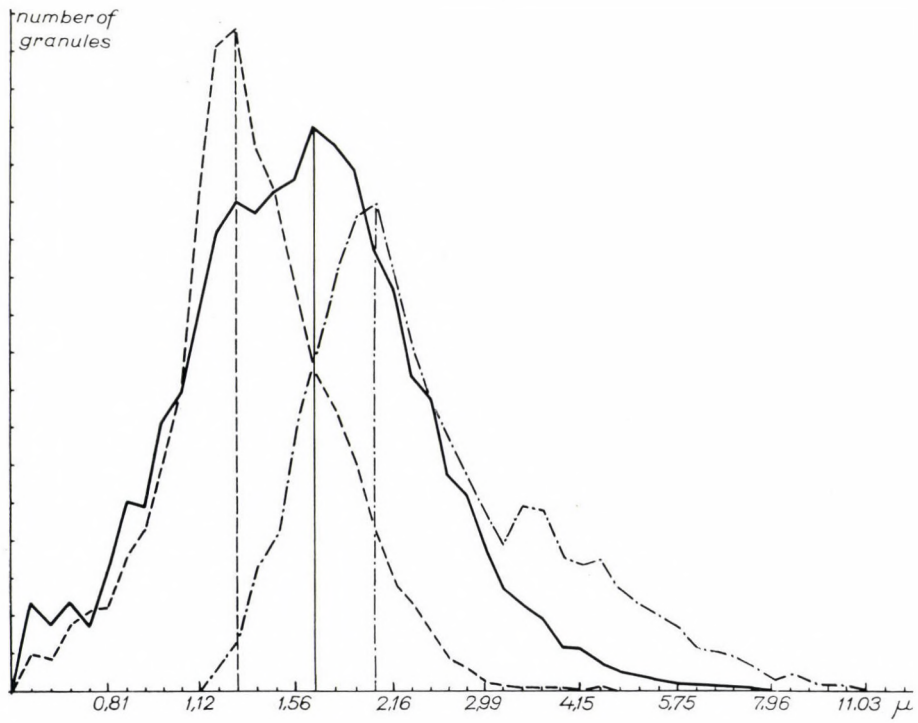

Fig. 2. Lipid vesicle distribution curves, plotted from native smears of infantile and adult glands and of chromodacryorrhoeic excretion. The maxima of the values are: for infantile gland, 1.29 (———); for adult gland, 1.66 (———); secretion, (· · · · · · · · · · ·) 2.02 μ.

For the electron microscopic studies 2 Harderian glands of the infantile and 2 of the adult group were embedded. 1000 vesicles per animal were counted in the ultrathin sections. The result for infantile animals was 1.05 ± 0.042 μ for adults 1.37 ± 0.057 μ.

Figs 3 and 4 show the electron microscopic view of the Harderian glands of infantile and adult animals, as well as the distribution curves obtained.

The curves are similar in shape to those obtained from the homogenates, but in both age groups the maxima are smaller by 3 orders of magnitude.

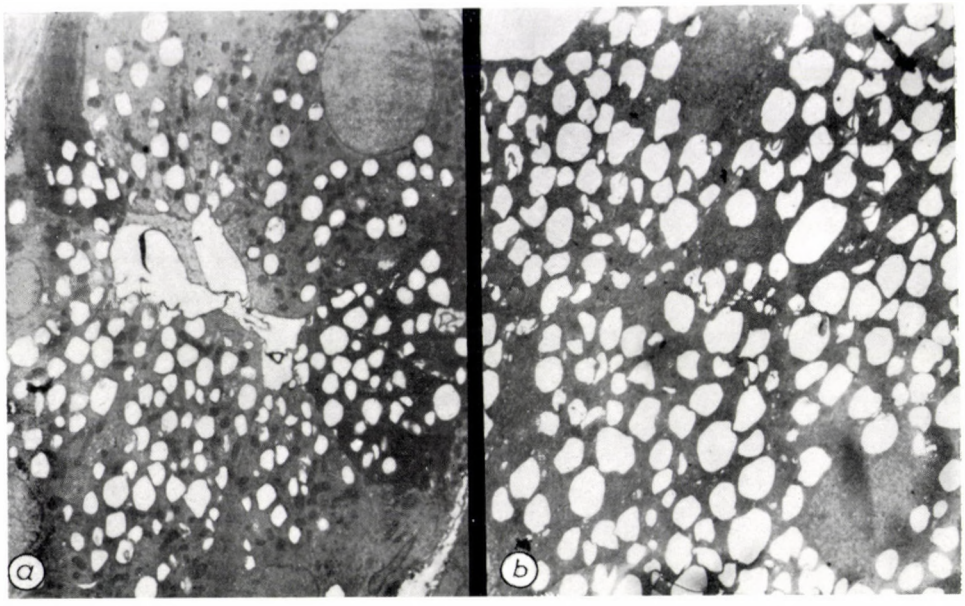

Fig. 3. Electron microgram of the Harderian gland of infantile (a) and adult (b) rat. Fixation with OsO_4, embedding in methacrylate, × 4000

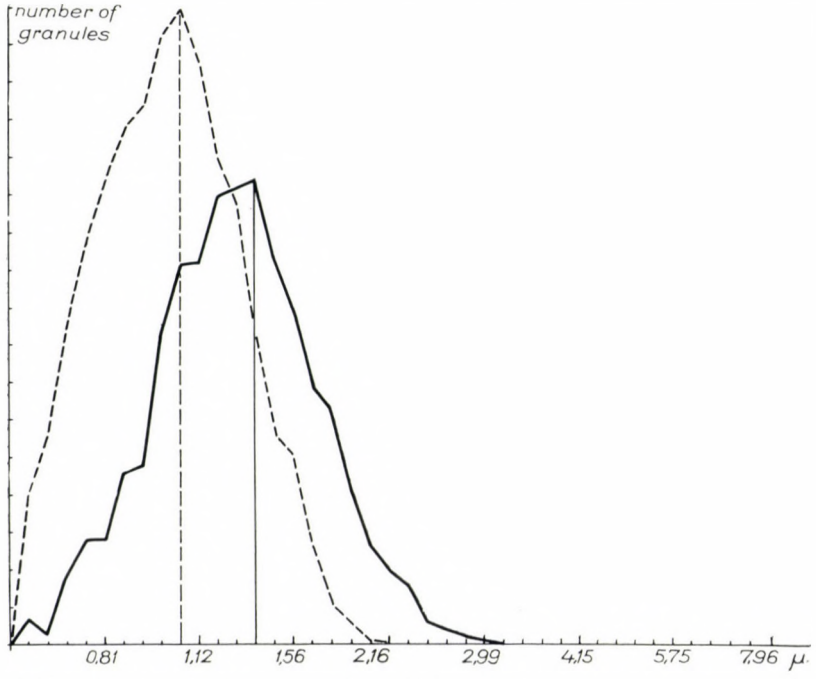

Fig. 4. Lipid vesicle distribution curves plotted from the electron micrograms of the Harderian gland of infantile and adult rats. Maxima: infantile animals, 1.05 μ (- - - - -); adult ones, 1.37 μ (———)

This difference amounts to about 0.3 μ and may apparently be due to tissue shrinkage resulting from fixation and embedding for electron microscopy.

In the second group of observations we analysed the picture of the vesicles in the smears prepared from chromodacryorrhoea induced by pilocarpine and choline chloride treatment, respectively. No significant difference could be detected in the response to these two parasympathicomimetics. Pilocarpine was injected into two adult animals weighing 170 and 190 g, and choline chloride into one animal. The distribution curves were plotted by measuring 1000 vesicles in each smear. The maximum of the values found was 2.02 ± 0.081 μ (see Fig. 2).

In the chromodacryorrhoeic discharge the larger vesicles are excreted, in the first place, while, as indicated by the ascending branch of the distribution curve, the vesicles of minute size are not excreted at all. The outflow of vesicles begins with those about 1.2 μ in diameter, whereas the vesicles of larger diameter, present in comparatively small quantities in the gland, occur in relatively larger numbers in the excretion. In each of the chromodacryorrhoeic curves there is a small peak in the 3.41 to 3.64 μ range, clearly visible in the overall curve, too. This presumably corresponds to the maximum of the mature vesicles, ready to be excreted.

Discussion

On the basis of experimental observations the conclusion has been arrived at that in parallel with the maturation of the Harderian gland the diameter size of the lipid vesicles of the glandular epithelial cells gradually increases. This phenomenon can be observed both under the light and electron microscope. However, the electron microscopic studies call attention to the fact that besides the gradual increase in vesicle size another factor: the increase in number of the vesicles should also be taken into account in the process of maturation of the glandular cells. Although no quantitative data are available in this respect, we still think that the two factors: the increase in size and number of the vesicles clearly define the process of glandular maturation. This observation seems to be in parallel with an earlier one made in this Department according to which in the gland of infantile animals the quantity of porphyrin bound to the unsaturated lipid membrane of the lipid vesicles is smaller than that in the glands of adult animals [6].

Summing up the morphological and functional evidence obtained thus far in connection with the Harderian gland, the function of the gland may be characterised as follows: the vesicles arising from the cytoplasmic Golgi apparatus, possessing an unsaturated lipid membrane, increasing gradually in size and number enclose the cytoplasmic components (lipids, porphyrin) and

in the course of secretion leave the glandular acini together with these components.

Many details of this process are not fully understood. So very little or nothing is known of the chemical structure of the lipid of the Harderian gland, of the cellular localisation of the biosynthesis of porphyrin, of the apocrine or holocrine nature of glandular secretion.

At this stage of our observations it is still questionable how the lipid vesicles increase in size. Earlier electron microscopic observations point to appositional growth from the cytoplasmic Golgi apparatus [5], but a fusion of single lipid vesicles may also be involved. Statistical analysis of the lipid vesicle diameters of the chromodacryorrhoeic excretions suggests that those are the larger vesicles in the first place that leave the glandular acini by chromodacryorrhoea. However, it should be emphasized that under the present experimental conditions chromodacryorrhoea can by no means be considered to be a physiological phenomenon, since the animals with rare exceptions are lost during the experiment: the phenomenon should rather be considered as supravital. For this reason no far-reaching conclusions can be drawn from the chromodacryorrhoea experiments, as to glandular function. Nevertheless, the circumstance that those are the vesicles of larger size that appear in the first place in the chromodacryorrhoeic excretion, the ones that are nearer to the luminar surface of the glandular epithelial cells, as determined by electron microscopy, suggests that the maximum of the increment in diameter observed during the maturation of the vesicles occurs in the secretory stage.

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UNTERSUCHUNG DER DURCHMESSER-VERTEILUNG IN DEN SEKRETORISCHEN LIPOIDTROPFEN VON HARDER-DRÜSEN

Die Durchmesser-Verteilung der sekretorischen Elemente, der Lipoidtropfen oder Vesiculae der Harder-Drüse wurde untersucht. Die Untersuchungsergebnisse lassen sich wie folgt zusammenfassen: In den nativen Drüsenhomogenisat-Ausstrichen, in den elektronen-

mikroskopischen Präparaten von infantilen und ausgewachsenen Tieren sowie im chromodacryorrhoeischen Sekret weisen die Lipoidtropfen- (Vesiculae-) Durchmesser erhebliche Unterschiede auf. Die Drüsen infantiler Tiere enthalten die kleinsten, die Drüsen ausgewachsener Tiere grössere, und das chromodacryorrhoeische Sekret die grössten Körnchen.

ИССЛЕДОВАНИЯ РАСПРЕДЕЛЕНИЯ РАЗМЕРОВ ДИАМЕТРА СЕКРЕТОРНЫХ ЛИПОИДНЫХ ЗЕРНЫШЕК ГАРДЕРОВОЙ ЖЕЛЕЗЫ

Авторы статистическим способом исследовали условия распределения размеров диаметра секреторных элементов, липоидных зерен или пузырьков Гардеровой железы. Согласно полученным результатам диаметры липоидных зерен (пузырьков) в нативных мазках гомогенизата железы, в электронномикроскопических препаратах и в хромодакриорейном секрете инфантильных и взрослых животных показали значительную разницу в том смысле, что железы инфантильных животных содержат наименьшие зернышки; размеры диаметра зернышек в железе взрослых животных больше, а наибольшие диаметры зернышек наблюдались в хромодакриорейном секрете.

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REGENERATION AND SOMATIC EMBRYOGENESIS OF ACTINIA EQUINA IN DIFFERENT STAGES OF ONTOGENETIC DEVELOPMENT

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Synopsis

Actinia equina L. in different stages of development (late larvae with 12 tentacles taken from the gastral cavity of the mother, young sessile individuals with 24 tentacles and adult forms of 3 to 4 cm diameter) were transsected at different levels of the body. Aboral pieces lacking oral structures were capable to restore them. Restoration was most rapid in the young animals. Removed pedal disc was regenerated only in larvae. The latter process was frequently accompanied by the formation of a second individual from the regenerated pedal disc. The results are discussed on the basis of TOKIN's theory on the dependency of regeneration and somatic embryogenesis on the level of integration.

Introduction

The dependency of regeneration phenomena and of somatic embryogenesis on the level of organization, age and physiological state of the experimental animal (i.e. on the level of its integration) is an actual and interesting problem of modern experimental embryology [17, 18]. The comparison of regenerative power and somatic embryogenesis within individual phyla is of special interest. It follows from TOKIN's theory [13, 17, 18] concerning the relationships of regeneration and somatic embryogenesis on one hand and the level of the organization on the other that decreased regenerative capacity is to be expected with aged organisms when the integration mechanisms controlling the life of the organism change. Peculiar forms of regeneration must occur in larvae if they are simpler and less integrated than the adults.

In this connection certain observations on changes in regenerative power during the ontogenesis of *Actinia equina L.* may be of interest.

Asexual reproduction and regeneration of anemones have been considerably studied [1 to 7, 9, 10, 14, 16 etc.] in view of the rich and various material presented by these animals. In many species of anemones more than one type of asexual reproduction are assumed to occur. CARLGREN [4 to 7], HAMMATT [11], TORREY [19] studied the effect of asexual reproduction on the morphology of *Metridium* supplying data on variation of siphonoglyphs, on the number of septa and general symmetry, further on the frequent formation of doubled monsters, assuming that *Metridium* reproduces by fission, budding

and laceration. In the case of *Actinia equina* laceration, budding and longitudinal fission are assumed as normal modes of reproduction in addition to viviparity. STEPHENSON [16], a recognized authority on this group, showed, however, that only one mode of reproduction is a specific characteristic of anemones while all other cases of asexual reproduction are but results of traumatisation of the animals under natural circumstances. TORREY [19] found the frequency of fission and budding in *Metridium* to attain but 2.5 per cent of the frequency of laceration. If e.g. budding is reported to occur with certain species it is actually nothing else than a supernumerary tuft of tentacles (sometimes with oral disk and pharynx) on one side of the animal. The formation of this structure is due to restoration after injury, i.e. to the process referred to as somatic embryogenesis [13, 17, 18]. In this context the different cases of heteromorphosis found in anemones should also be mentioned [4, 5, 6, 12, 14]. CERFONTAINE [8] found e.g. that pieces of transversely cut *Asteroides calycularis* form tentacles, mouth and pharynx on the aboral side. HAHN [10] ascribed dimorphism, i.e. the existence of animals with one or two directives in *Metridium* to asexual reproduction. This occurs spontaneously by means of basal fragmentation and can be induced by cutting pieces from the pedal disc. The type (monoglyphic or diglyphic) of the developing polyp depends on the presence or absence of a part of the directive in the fragment because there is an obligate neoformation of this system in regenerating tissues of young polyps. The formation of animals with two directives which developed simultaneously in the regenerating tissue and with separate oesophagi, present the explanation of the occurrence of doubled monsters and triglyphic polyps. It is evident that these are cases of somatic embryogenesis. The formation of a second directive in the separated piece in addition to the existent one which frequently leads also to the formation of a new pharynx is nothing else than a manifestation of the tendency to form a new organism. Accordingly the ways of natural reproduction of anemones are not so various as it is generally assumed because a considerable part of the reported cases represent in reality cases of asexual reproduction artificially induced (by injuries), i.e. somatic embryogenesis.

STEPHENSON [16] proposed the sexual reproduction (viviparity) to be regarded as the normal way of reproduction in *Actinia equina*.

Anemones possess a well known high regenerative power. Certain observations [1 to 7] revealed, however, that complete animals regenerated only from fragments containing but a small part of the pedal disc. During normal asexual reproduction, most frequently by longitudinal fission or pedal laceration, the daughter individuals necessarily obtain parts of the pedal disc. An interesting exception from this rule constitute the more primitive genera *Protanthea* and *Boloceroides* which are probably less integrated and have a more simple morphological organization. In *Protanthea* a transverse fission

with furrow formation is observed producing an upper half without pedal disc which, however, develops into a complete organism.

CARLGREN [7] made a comparison of developmental processes in isolated parts of the column wall in *Protanthe* and *Metridium*. The first species forms tentacles and pharynx in this case, while the latter only tentacles. These differences were attributed to different levels of organisation. *Boloceroides* is capable of autotomy of tentacles which, being separated, give again complete animals [15]. Accordingly, the presence of the pedal disc or part of it is an

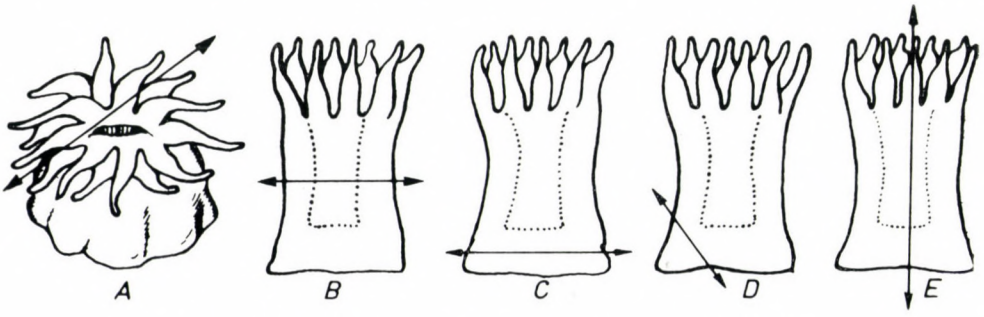

Fig. 1. Scheme of operations performed on adult animals

obligate condition for the development of complete organism in *Actinia equina* and in most anemones. The physiological basis of this phenomenon is unknown.

The results of our earlier unpublished experiments with different pieces of adult *Actinia equina* are in complete accordance with the above discussed literature. Basal parts of the body easily restored removed tentacles (Fig. 1 *A*) or the distal body region (Fig. 1 *B*). A complete or incomplete pedal disc gives rise to complete organisms (Fig. 1 *C* and *D*). In certain experiments the pedal disc isolated by a horizontal cutting and then divided into four sectors give four complete organisms. Longitudinal cutting produced two organisms (Fig. 1 *E*).

In the present work the behaviour of oral and aboral parts obtained by transverse cutting of animals in different ontogenetical stages were studied in view of possible differences in the regeneration due to different levels of integration.

Materials and methods

The experiments were performed on *Actinia equina L.* collected from the Barents sea.

To study the ontogenetic differences in the regeneration, animals in the following stages were used:

(1) Late larvae taken from the gastrovascular cavity of the mother. Their pedal disc did not function yet and was morphologically not quite distinct. Its diameter was 1 to 1.5 mm. In most cases two rows of tentacles (6 large and 6 small) were present.

(2) Young animals which have left the body of the mother and were attached to the bottom of the aquarium. They had 24 tentacles and a pedal disc of 5 to 7 mm diameter.

(3) Adult animals having not less than 48 tentacles. It is difficult to state their age but they were the largest available specimens with a diameter of 3 to 4 cm.

Transverse cuttings were made at three levels (Fig. 2), only the oral disc and the tentacles were cut off (I), the mid part and the pharynx were cut (II) or only the pedal disc with the attachments of septae was left (III). Each set of experiments included about 40 animals. The operated individuals were kept in aquaria fed with running sea water of 6 to 8° C. The animals were regularly checked and drawings were made in living state. Some animals were fixed and sectioned. The maximal span of postoperative follow up was 45 to 50 days.

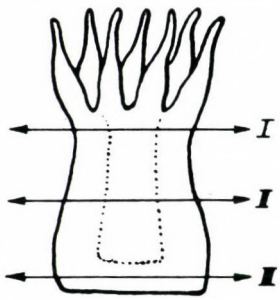

Fig. 2. Levels of transverse transections on anemones in different stages of ontogenetic development

All the experimental work was executed in the Murmansk Biological Institute of the USSR Academy of Sciences in June and July, 1957, 1958 and 1960.

Results

Tables 1 and 2 present the results of the experiments. The basal part of the body in all experiments invariably restored the removed oral part but the duration of the regeneration was different. Table 1 shows the time (mean values with individual differences of 3 to 4 days) necessary to the appearance of clearly visible anlagen of tentacles on the newly formed oral disc. Table 1 shows a direct relationship between the period of restoration and the size of the removed part.

Table 1

Duration (in days) of complete regeneration of Actinia equina *from the basal part of the body*

Stage / Level of cutting	Larval	Young	Adult
I	19	11	16
II	23	16	25
III	30	19	45

The relationship between regeneration and age of the animal proved to be more complex. Fastest regeneration was found in young animals with 24 tentacles (11 to 19 days after different types of cuttings); it was much slower in late larvae (19 to 30 days) which is most probably due to less pronounced integration of the larvae. Still slower restoration of the oral part is observed with adult specimens (16 to 45 days) which can be most probably explained by pre-sumable occurrence of old age changes leading to elimination of certain integrative mechanisms. Mechanophysiological phenomena (changes in turgor, weaker sphincters which close the wounds) might also be involved. The fate of the distal parts was rather varied in different experiments (Table 2).

Table 2

Fate of oral parts of Actinia equina *after cutting*

Stage / Level of cutting	Larval	Young	Adult
I	Survive for 45 days; basal disc not restored	Survive for 45 days; basal disc not restored	Die after 6 days
II	The same; may laterally attach for a certain time	The same	Die after 23 days
III	Complete regeneration of basal disc	Commencement of wound healing	Survive for 45 days; basal disc not restored

Distal parts of adult animals never showed any sign of regeneration. After level I or II cuttings oral parts died and started to decay after 6 to 23 days. After level III cuttings most part of the body survived for 45 days and responded to mechanical stimulation with slight changes of body form. The contraction of the circular muscles closed the wound but no formative processes could be observed on the wound surface. The greater viability of the oral parts after level III cuttings is certainly explained by their size. In fact they are almost complete individuals with only the pedal disc lacking.

The oral parts of young animals, with 24 tentacles, show the same behaviour after level I cuttings: they survive for long time but show no signs of development.

After level II transection the oral parts of young animals were in a better condition. In spite of the absence of basal disc regeneration they may adhere to the substrate with the lateral body wall. The viability of young animals after level III cuttings is still higher. The fragments show an increase in size (presumably they feed), a membrane covers the wound faintly reveal-

ing the septa. No pedal disc is, however, formed and the animals attach to the substrate with their lateral side, as in the previous case.

The results of experiments with larvae proved to be the most interesting. Subsequently to level I transection the oral part survived for 45 days but no pedal disc was formed. Oral parts obtained by level II cuttings show similar behaviour as found in young animals after level II or III transections.

The oral part of larvae obtained by level III transection showed, however, signs of progressive development. The wound is closed by muscular contraction

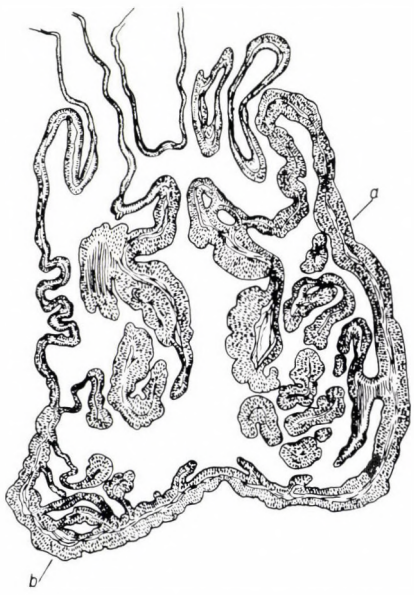

Fig. 3. Schematic longitudinal section through animal with "bud". *a* — original individual, *b* — "bud"

at first and by growth of the ecto- and endoderm later. A genuine pedal disc with well formed musculature develops serving the attachment to the substrate. Accordingly, complete small animals are formed in this case from the oral fragment which was never observed with more advanced stages. Presumably the physiological dominance of the pedal disc over other body regions is less pronounced in this stage of development.

In 8 cases out of 45 level III cuttings on larvae (i.e. 17.8 per cent) on the margin of the newly formed pedal disc a small bud-like appendix appeared. On longitudinal sections (Fig. 3) the "bud" is seen to represent an outgrowth of the body wall with forming radial entodermal folds, i.e. septa. They run in variance to the mother organism not toward the pharynx but to each other. The subsequent fate could not be observed due to short range of the experiments performed in 1958.

Two years later the latter experiments were repeated on 35 larvae which were followed up for 50 days. 30 animals of 35 survived until the end of the observation period. On the 3rd to 4th day all specimens attach themselves to the bottom of the vessel with the mucus-covered body walls. On the 10th day the wound is closed under the mucus by entodermal and ectodermal layer creeping from the periphery to the center. A layer of mesogloea invades between the two layers.

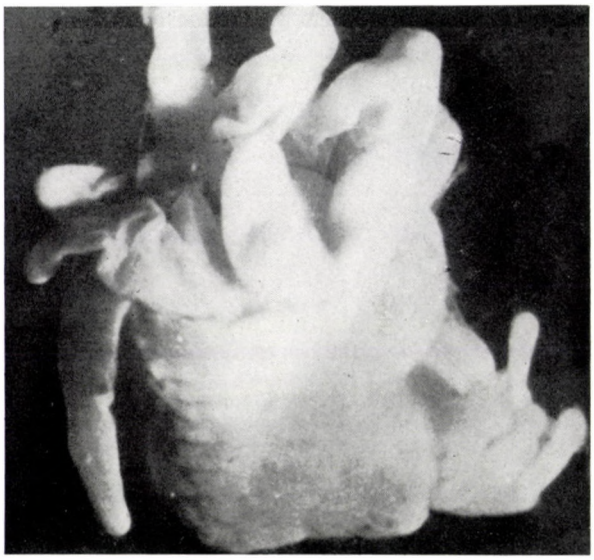

Fig. 4. Somatic embryogenesis of larval *Actinia equina*

The first "buds" appear two weeks after operation. In the 5th week these processes formed on the aboral side of the animals already have an oral disc with a mouth and a circlet of tentacles, i.e. they represent a new individuum (Fig. 4). Thus no typical regeneration, but somatic embryogenesis, i.e. artificial asexual reproduction was obtained in this case.

The developing daughter animal tends to assume a vertical position. The oral discs of both individuums approach and on a common pedal disc there are two columns both carrying oral discs with tentacles. The tentacle number is different — if the mother has 12, then the daughter has only 6 which are also thinner and smaller.

The formation of the tentacles repeats the events of embryogenesis, at first the first circlet (6) and then the second one is formed. There are no differences in septa formation either.

The new oral part is as active as the old one, it captures the pray with its tentacles and on stimulation the whole oral disk with the tentacles withdraws into the body.

Fig. 5 shows a scheme of a longitudinal section through an animal with accessory mouth, pharynx and tentacles. Fixation unfortunately led to contraction which obscured the new column and the tentacles are also drawn into the pharyngeal cavity. The new oral opening with the pharynx leading to the old one can be, however, clearly seen.

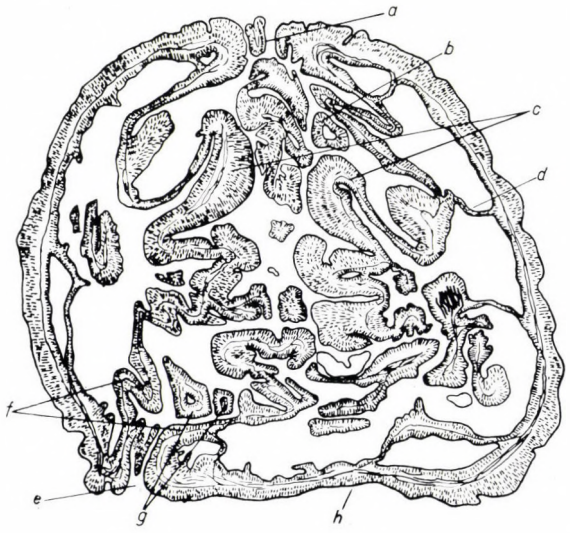

Fig. 5. Longitudinal section of an animal with accessory mouth and pharynx. *a* — mouth, *b* — tentacle, *c* — pharynx and *d* — septa of the mother organisms, *e* — mouth, *f* — pharynx and *g* — tentacle of the daughter organism, *h* — regenerated pedal disc

In 18 animals out of 30 survivors (i.e. in 60 per cent) was somatic embryogenesis observed.

A further interesting result was the appearance of well developed pedal discs. These carried the "doubled" animals which were formed as a result of somatic embryogenesis or single forms in which the formation of pedal disc was the only result of the regeneration.

Discussion

The level of complexity and of integrity of organisms may be different in various stages of ontogenetic development. Therefore their capacity to regenerate or to undergo somatic embryogenesis will also change [13, 17, 18].

Young anemones which were completely formed and were attached to the substrate show the most rapid regeneration. Adults and larvae regenerate more slowly. The larvae of *Actinia equina* can produce new pedal disc which is never observed with developed individuals. The removal of the pedal disc of larvae is not an irreparable damage (as it is in more advanced stages of development).

The work of ABELOOS [1] on restoration of *Actinia equina* from pedal disc should be referred to in this connection. If the pedal sac (the first result of regeneration from pedal disc) is transversely cut at the beginning of its regulation, the aboral fragment regenerates again an oral part, the oral part, however, changes and forms a pedal disc. ABELOOS attributes the absence of regeneration of pedal disc from normal column wall to the lack of histogenetic potency in the latter. We are inclined to assume that the phenomenon might be interpreted as a consequence of stronger integrity and slighter lability of parts of the fully formed animals as compared with larvae and with early regeneration stages of an isolated pedal disc.

The larvae of *Actinia equina* are capable to undergo somatic embryogenesis [13, 17, 18]. At the end of the larval period when the pedal disc does not function and is only slightly distinct, a local desintegration and a consequent tendency to form a new individual is easily produced in the aboral region. This points to weaker relationships of the parts of the larvae i.e. to its weaker integrity as compared with adult forms.

Presumably several cases of somatic embryogenesis were reported in the literature as modes of natural asexual reproduction.

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REGENERATION UND SOMATISCHE EMBRYOGENESE IN VERSCHIEDENEN STADIEN DER ONTOGENESE VON ACTINIA EQUINA

Actinia equina L. wurde in verschiedenen Stadien der Entwicklung (junge Larven mit Tentakeln aus dem Gastralraum der Mutter, sessile Jungtiere mit 24 Tentakeln und adulte Tiere mit einem Durchmesser von 3 bis 4 cm) in verschiedenen Ebenen zerschnitten. Aborale Teile, die keine oralen Strukturen besassen, konnten diese neubilden. Diese Neubildung verlief am schnellsten bei jungen Tieren. Entfernte Fussscheiben konnten nur Larven regenerieren. Im weiteren Verlauf dieses Prozesses wurde von der regenerierten Fussscheibe häufig auch ein zweites Individuum gebildet. Die Resultate werden auf Grund der TOKINschen Theorie über die Abhängigkeit von Regeneration und somatischer Embryogenese vom Grad der Integration behandelt.

ЯВЛЕНИЯ РЕГЕНЕРАЦИИ И СОМАТИЧЕСКОГО ЭМБРИОГЕНЕЗА В ОНТОГЕНЕЗЕ ACTINIA EQUINA

Actinia equina L. на трех стадиях онтогенеза (поздние зародыши, имеющие 12 щупалец, извлеченные из гастро-васкулярной полости матери; молодые актинии, снабженные 24 щупальцами и ведущие прикрепленный ббраз жизни и взрослые актинии диаметром 3—4 см) разрезались поперек на трех уровнях тела. Базальная часть регенерировала удаленную оральную часть. Быстрее всего этот процесс осуществляется у молодых актиний. Удаленная подошва восстанавливается только у зародышей. Этот процесс часто сопровождается развитием дочерной актинии из восстановленной подошвы. Автор обсуждает результаты с точки зрения теории *Токина* о зависимости регенерации и соматического эмбриогенеза от уровня интеграции.

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Acta Biol. Hung., **14** (3), 209—213 (1963)

STUDIES ON FEEDING AND DIGESTION IN PROTOZOA

VI. THE EFFECT OF INGESTION OF NON-NUTRITIVE PARTICLES ON ACID PHOSPHATASE IN PARAMECIUM MULTIMICRONUCLEATUM

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(Received August 1, 1963)

Synopsis

Paramecium multimicronucleatum taken directly from the culture or starved for a day takes up more or less readily different non-nutritive particles (iron particles, calcium carbonate, talc, China ink, carmine) and forms vacuoles containig exclusively these particles. The vacuoles invariably show histochemically demonstrable intense acid phosphatase activity. The results support the view that the appearance of activity in vacuoles is not dependent on the nature of ingested substances but on the vacuole formation itself.

Introduction

Food vacuoles of protozoa containing nutritive particles are known to exhibit a strong activity of histochemically demonstrable hydrolytic enzymes (for reviews see [1, 13]). It is assumed that these enzymes participate in the breakdown of ingested materials [4]. Rosenbaum and Wittner [16] recently reported that no enzyme activity was observed in *Paramecium caudatum* vacuoles which contained only non-nutritive, indigestible substances (carmine or China ink particles). Müller and co-workers [12, 13] have found, however, that vacuoles of *P. multimicronucleatum* containing polystyrene latex particles exhibit an activity not inferior to that of vacuoles in animals fed with bacteria. The discrepancies in these results prompted us to test if there were any differences in reactions of paramecia to different types of non-nutritive particles. The present communication contains information concerning animals which ingested different kinds of non-nutritive particles. Polystyrene latex does not figure among these. The uptake of and reactions to the polystyrene particles are dealt with in a separate communication [12] where also the significance of the results obtained is discussed in detail.

Materials and methods

Paramecium multimicronucleatum, a Hungarian clone, used also in previous studies, was grown in a crude agnotobiotic culture on diluted manure infusion. The animals were collected with the aid of a hand driven centrifuge, repeatedly washed and resuspended in

Losina-Losinsky solution [9] without peptone. The animals were used either immediately after washing or kept in large amounts of this solution for one day before the experiments were carried out. This period of time appeared to be long enough for obtaining a very high percentage of vacuole free animals.

The animals were given different inorganic and organic substances as non-nutritive particles. The substances used were: iron particles (Ferrum reductum Ph. Hung. V), calcium carbonate (Calcium carbonicum precip. Ph. Hung. V), powdered talc (Talcum pulvis), powdered glass (prepared in a ball mill), China ink (obtained by grinding a rod), carmine (Carmin rubrum optimale). Heavy suspensions of animals were placed in depressionsli des and a comparatively large amount of the respective substance was added. The duration of feeding was 30 minutes.

The histochemical reactions were studied in total mounts. A drop of fluid containing a large amount of animals was spread on an albuminized slide and rapidly dried in an air current. The slides were fixed in cold Ca-formol for 15 to 30 min. washed in tap water and mounted in balsam or glycerine jelly after performing the histochemical reactions. Acid phosphatase activity was demonstrated with the lead phosphate method of GOMORI [15] at pH 5.0 and 30° C or with the alpha-naphthyl phosphate-hexazotized rosanilin method of BARKA [2] at pH 6.0 and 25° C.

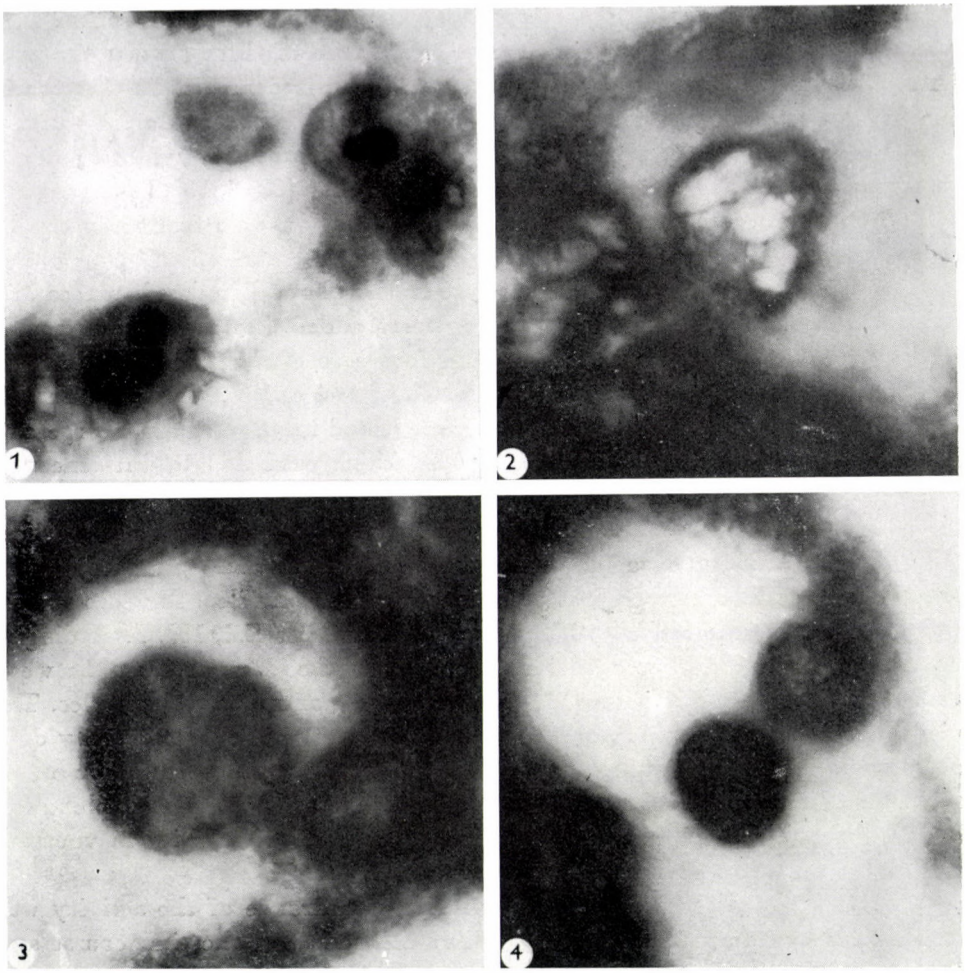
Results

Animals studied immediately after washing contained a large number of bacterium filled food vacuoles. Acid phosphatase staining revealed activity in most of the vacuoles and in a moderate amount of cytoplasmic granules. Those animals which had been starved for 24 hours were either vacuole free or contained only a very small number of small vacuoles. After acid phosphatase reaction these animals showed a darker cytoplasmic staining than the former ones. The infrequent individuals containing vacuoles showed intense vacuolar activity visible as small circles.

Both washed and starved animals ingested most kinds of the particles tested. Vacuole formation was rapid in the case of calcium carbonate, talc, China ink and carmine. The larger and heavier iron particles were less readily accepted. Only the glass powder was rejected by most animals. In certain animals, however, glass vacuoles also appeared. The number of these individuals was not enough for histochemical evaluation.

Histochemical reactions performed on starved individuals which took up iron, calcium carbonate, talc or carmine clearly revealed an intense enzymatic activity in most large vacuoles containing the non-nutritive substances (Figs. 1 to 4). Even in animals which were not completely vacuole free the preexistent small vacuoles and the newly formed larger ones could easily be distinguished. The activity resulted in a periferic staining of the vacuole, i.e. most probably the vacuolar wall was stained. Vacuoles formed in animals fed on China ink were so dark that their black content completely obscured the picture and it could not be decided if any staining was present.

Similar activity was found in vacuoles filled with non-nutritive particles when they were formed by not starved animals which contained numerous bacteria filled vacuole, too.



Figs. 1 to *4.* Acid phosphatase reaction according to BARKA in vacuoles containing exclusively particles of iron (Fig. 1), calcium carbonate (Fig. 2), talc (Fig. 3) and carmine (Fig. 4) formed by *Paramecium multimicronucleatum* which starved for 24 hours. Total mounts, × 1750

Discussion

The ingestion of non-nutritive, indigestible substances by different species of *Paramecium* was repeatedly reported either in papers concerning feeding in this animal or in communications on other physiological problems. The following list of ingested particles presents only certain examples and is far from complete: sulphur [5], iron powder [8], aluminium powder [10], calcium carbonate, stroncium carbonate, magnesium carbonate, barium carbonate [5, 9], barium chromate [6], glass powder [3, 5, 10], China ink [9, 10, 16], carmine [9, 10, 16], indigo [9, 10], sepia [10], polystyrene latex

[12, 13]. The results of all these studies clearly show that non-nutritive substances are readily ingested by paramecia. In some of these studies the non-nutritive particles were added to animals in bacterized media. In these cases the vacuole formation might be due to the bacteria present. In a fair number of studies, however, the particles were added to thoroughly washed animals showing no vacuole formation without added particles [3, 12, 16]. Food vacuoles appearing under such conditions demonstrate that vacuole formation can be induced by non-nutritive particles.

In the present experiments several of the above listed substances and some which were not employed formerly were added to washed and vacuole free animals. All substances tested were found to evoke vacuole formation thus confirming the view outlined above. The speed of vacuole formation and the number of vacuoles were completely neglected in view of those large and non-controllable differences in these values which were due to differences in the size and other properties of the particles.

The results of histochemical tests performed in this work clearly show that intense activity of acid phosphatase can be localised in paramecium food vacuoles which contain but only non-nutritive particles. The same result was obtained in animals fed with polystyrene particles [12, 13]. Accordingly, no fundamental differences in this respect can be demonstrated between vacuoles containing nutritive [11, 13, 14, 16] or non-nutritive particles. The view suggested in other papers [12, 13] that the appearance of activity does not depend on the contents of the vacuoles but only on their formation seems to be further supported by the present observations. So far no explanation can be given for the reports concerning the absence of activity in vacuoles containing only non-nutritive substances [16].

In the present communication only the appearance of the activity was emphasized and discussed and the possible differences in the time course and in the level of hydrolytic activity are not considered. No doubt, the existence of such differences due to the nature of the ingested particles can be proved or disproved only by biochemical studies.

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ÜBER DIE NAHRUNGSAUFNAHME UND VERDAUUNG BEI PROTOZOEN

VI. Die Wirkung der Aufnahme von unverdaulichen Partikeln auf die saure Phosphataseaktivität in Paramecium multimicronucleatum

Paramecien, die entweder direkt der Kultur entnommen wurden oder einen Tag lang hungerten, nehmen verschiedene unverdauliche Partikeln (Eisen, Kalk, Talkum, Glas, Tusche und Karmin) mehr oder minder schnell auf und bilden Vakuolen, die ausschliesslich diese Substanzen enthalten. Diese Vakuolen zeigen eine starke, histochemisch nachweisbare Aktivität von sauren Phosphatase. Dieser Ergebnis unterstützt die Annahme, dass das Erscheinen der Aktivität in der Vakuole nicht von der chemischen Beschaffenheit der Partikeln, sondern von der Ausbindlung der Vakuole abhängig ist.

ЗАХВАТ ПИЩИ И ПИЩЕВАРЕНИЕ У ПРОСТЕЙШИХ

VI. Влияние заглатывания непереваримых частиц на активность кислой фосфатазы у Paramecium multimicronucleatum

Paramecium multimicronucleatum взятая непосредственно из культуры или голодающая на сутки усваивают более или менее охотно разные неперевариме частицы (железа, карбоната кальция, талькума, стекла, туши и кармина) и образовывают вакуоли, содержащие исключительно эти частицы. Эти вакуоли имеют высокую, гистохимически доказаемую активность кислой фосфотазы. Результаты подкрепляют мнение о том, что появление активности в вакуолях не зависит от химической природы частиц, а связана с появлением вакуоли.

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HERITABILITY OF THE FACTORS OF NATURAL IMMUNITY IN RATS

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Synopsis

Investigating the genetic conditions of natural immunity, we have pointed out that rats belonging to different varieties (grey, laboratory white and Wistar) differ from one another regarding their immunobiological titres — namely complement titre and bactericidal properties — of their sera. Serum of grey rats had the highest and that of laboratory white ones the lowest complement titre and bactericidal activity. When Wistar and grey rats were crossed, the complement titre and bactericidal power of the hybrids' sera showed intermediate values as compared with the titres of the parents.

It also has been pointed out that, similarly to the complement titre and bactericidal activity, the sera of grey rats contained the highest amount of properdin, while sera of laboratory white rats the lowest. It may be supposed therefore that properdin level, and consequently factors involved in the formation of natural immunity, are different not only in species, but also in varieties.

Crossing of grey and laboratory white rats showed a surprising result. While the average properdin content in the grey rats' sera was 36.6 U/ml and that of laboratory white ones 18.3 U/ml, the sera of hybrid animals contained 48.3 U. of properdin per ml. On the strength of these results it may be suggested that immunobiological titres are determined by genetic factors, and heterosis effect may occur in them. On the other hand it is evident that environmentalfactors (such as exposure to cold) can change the degree of immunity within a com paratively short period.

Introduction

It is a well known fact that animals belonging to various species differ from each other as to their immunobiological titres. This has been pointed out for example by HEGEDÜS and GREINER [3] as well as PILLEMER and coworkers [6] in respect to the complement and properdin, by proving that human serum contains 80 units, while sera of guinea pigs 200, rabbits 20, rats 100, pigs 50, dogs 40 units of complement per ml., and serum of cattle, horses, sheeps and gophers *(Spermophilus)* have no complement activity at all. Not only the total complement, but also its components may differ among species; to mention only one example, the serum of pigs contains 100 units of C'1, 800 units of C'2, 12,500 units of C'3 and 50 units of C'4 per ml. As related to the properdin this difference is also extent. Sera of rats contain 25—30, sera of mice 10—20, cattle 10—20, man and rabbit 4—8, sheep 2—4 and guinea pigs 1—2 units of properdin per ml. KENT, TOUSSAINT and HOOK's [4] data differ from these findings. They established that human sera contain

4—8, while sera of guinea pigs 8—12 units of properdin per ml. On the other hand CSEH and SZABÓ [1] who used a modified method for determining properdin found 2 units of it in a ml. of guinea pigs' sera, 8 units in rabbits' and 12 units in pigs' sera.

From these findings it may be inferred that the immunobiological properties and consequently the resistance against infections of the various species have evolved in the course of historical development and are hereditary characteristics.

Our own experiments, however, have clearly shown that the effect of environment, namely acclimatization to cold, may raise the complement titre of guinea pigs and that such a rise is accompanied by an increased bactericidal capacity of the serum as well as an increased phagocytic activity of the leucocytes [2, 9, 11]. It was also found that none of these changes — except increased phagocytosis — disappeared after the animals had been transferred to and kept in normal environment for a year or longer.

In earlier publications we have already emphasized that the principal object of our experiments was to elucidate the problem of heredity of natural immunity [2]. It is the way in which living organisms have developed the power to resist infections that we tried to find out, and to ascertain the role of hereditary factors in such developments.

From our experiments it appears that properties acquired in the course of acclimatization to cold determine the degree of immunity and are transmitted by heredity more or less unchanged to the first generation. It may be assumed that the great differences in susceptibility of various species to infections are due to the inheritability of immunological factors, and that some species must at some time in the past have lived under conditions altering the titre of immune factors more radically, while others were not subjected to such conditions. This assumption seems to be confirmed by the well known fact that persons (and their descendants) who (and whose ascendants) had much to suffer from exposure to climatic vicissitudes are more resistant to infectious diseases than persons whose forebears were comparatively more protected. It is highly probable that good results obtained from raising calves and other young domestic animals in open air are likewise due to phenomena of this kind.

Consequently, environmental factors should be taken into consideration in the development of immunity.

Our results obtained by comparison of immunobiological values of different varieties within the same species deserve special interest [2, 10]. It has been found in our experiments that from grey, laboratory white and Wistar rats the grey rats had the highest complement titre, bactericidal capacity and even properdin level, while the serum of laboratory white rats had the lowest titres. This difference was considerable as related to bactericidal

activity (48 per cent), grey rats showing a 24 per cent higher, and laboratory white ones a 24 per cent lower bactericidal capacity as compared with the mean value. It is remarkable that serum of rats belonging to the Wistar stock stood very close to that of grey rats in respect of bactericidal capacity.

From the above mentioned results it is obvious that the natural immunity factors studied are present to different extent in the various varieties, grey rats possessing the highest amount of these factors and laboratory white ones the lowest. The favourable values of grey rats did not considerably decrease when they were kept or even born in captivity. Special interest is due to the fact that rats of Wistar stock approached, if not reached, the complement and bactericidal values found in grey rats. The importance of this finding lies in revealing that this widely used strain exhibits — besides its other well known good properties — its advantages in this respect too. This property of Wistar rats is probably connected with the higher protein requirement of these animals and should be taken into consideration when evaluating immunobiological and even metabolic tests carried out with this variety.

These results confirmed our belief that the factors of natural immunity are developed both by heredity and environmental influences, for the much lower immunobiological values of laboratory white rats domesticated long ago as compared with those of their wild relatives can certainly not be accidental. The relatively higher values of Wistar rats may be due to conselection or accumulation in the course of inbreeding.

Assuming that the heredity of natural immunity could be elucidated, we examined the properdin level in the sera of hybrids of grey and laboratory white rats and obtained the following results.

Materials and methods

The animals studied were bred in our own laboratory. Ordinary grey rats kept in the laboratory at least for a year after having been captured were crossed with laboratory white rats; albino males with grey females on the one hand and grey males with albino females on the other. Hybrids were raised and at the age of 3 to 5 months the properdin level of their sera was examined.

Blood for the experiments was drawn from male animals only by cutting an about 4 mm piece of their tails with sharp scissors in ether narcosis. The outflowing blood was collected in centrifuge tubes. Immediately after clotting the blood was centrifuged, the serum drawn off and kept overnight at -20° C in a deep freezer.

The determination of properdin was based on its well known ability to combine with zymosan, an insoluble carbohydrate derived from yeast cell walls, to form complex (PZ) that inactivated the third component of complement (C' 3) at 37° C.

So when zymosan-treated and zymosan-free sera were simultaneously incubated and a C' 3-free hemolytic system (sensitized sheep erythrocytes treated with R'3) was added, the zymosan-treated serum showed a decrease of hemolytic activity and from the degree of this decrease, after photometric evaluation, the absolute amount of properdin originally present in the system could be computed.

Owing to the drawbacks of the various methods for the determination of properdin originally described by PILLEMER and others [5, 6, 7, 8], and the difficulty in obtaining reagents, a method developed by CSEH and SZABÓ [1] was used. The reagents required (zymo-

san, RP and R'3 sera) were prepared in this laboratory. The scheme of the technique was as follows.

0.3 ml of buffered salt solution containing 0.003 mol veronal, 0.002 mol veronal sodium, 0.0005 mol magnesium chloride and 0.00015 mol calcium chloride per litre was filled into each of six test tubes. To the first and third tubes 0.3 ml each of the test serums were added. From these two tubes amounts of 0.3 ml each were diluted into the second and fourth tubes according to Widal's method. After adding 0.3 ml of RP (Properdin deficient) serum to each tube and 0.1 ml of zymosan solution to the first, second and fifth one, the content of each tube was brought up to 1 ml by buffered salt solution. The tubes were then shaken and incubated at 37° C in a water bath for 30 minutes and after cooling to 0° C and separating by centrifugation the C' 3 activity of the supernatant fluid was determined.

Results and discussion

The properdin titres of 24 rats from F_1 and 20 from the F_2 generation were examined and the following results obtained.

The properdin level of the F_1 rats ranged from 34.5 U/ml to 68 U/ml (average: 48.31 U/ml). Separate evaluation of the properdin levels of the descendants of white mothers and grey fathers and vice versa revealed that the direction of crossing does not essentially influence the results, as the sera of rats born from laboratory white mothers and grey fathers contained 34.5 to 65 U/ml of properdin (average: 48.56 U/ml), while sera of rats from reciprocal crossing contained 37.5 to 68 U/ml (average: 48.07 U/ml).

Sera of hybrid rats from the inbred F_2 generation showed similar properdin values. The properdin values of the animals examined were found to range 30 U/ml to 58.5 U/ml (average: 43.67 U/ml).

Comparing these data with the properdin titres of the different varieties, the properdin level of hybrid rats was found to be higher even than the titres of grey rats though in the original examination the latter had the highest values (from 30.5 U/ml to 40 U/ml).

From these surprising results the following inferences may be drawn:

(1) Though the results of our earlier examinations [2, 9, 10, 11, 12] showed that the changes in properdin and complement titres were closely correlated, factors causing the shift of complement titre altered the properdin titre too, and so the degree of natural immunity was strongly influenced by environmental conditions, nevertheless the properdin level of serum proved to be an inheritable quantitative character.

(2) From the former statement it is also obvious that the change of complement and properdin titres run parallel with each other only as long as this change is influenced by environmental factors, but their heredity is independent from each other. This seems to be supported by the well known fact that an animal species having a high complement titre (as for example guinea pigs) may not necessarily have a high properdin content in its serum as well.

(3) It may be established that by means of crossbreeding we have succeeded to produce animals having the highest level of this important immune globuline (i.e. properdin) in their sera among all the species hitherto examined.

(4) Consequently it may be suggested that producing animals of higher properdin level by the joint effect of crossing on the one hand, and environment on the other, may be an efficient and workable means of obtaining purified properdin.

(5) On the strength of our results it may be stated that heterosis effect might have a role in the heredity of immunobiological characteristics. These results may supply an explanation to the well known fact that vitality and resistance of hybrid animals to the deleterious effect of environment and consequently their chance of survival during raising are much better than those of the purebred ones. Probably the excellent properties of hybrid animals, especially their high resistance to infections are causally connected with the relatively high properdin level of their serum.

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GENETISCHE UND UMWELTFAKTOREN IN DER ENTWICKLUNG DER NATÜRLICHEN IMMUNITÄT VON RATTEN

Verfasser untersuchte in früheren Arbeiten die genetischen Beziehungen der natürlichen Immunität und stellte fest, dass die immunbiologischen Titer, besonders der Komplementtiter und die bakterizide Aktivität bei verschiedenen Rassen der Ratte (wilde Ratten, Albino- und Wistarratten) unterschiedlich sind. Den höchsten Komplementtiter und die grösste bakterizide Aktivität hatten die wilden Ratten, am niedrigsten waren die Werte bei den Albinos. Die Hybriden einer Kreuzung zwischen Wistar- und wilden Ratten zeigten einen intermediären Wert in bezug auf Komplementtiter und bakterizide Aktivität. Es gelang auch nachzuweisen, dass ebenso wie beim Komplementtiter und bei der bakteriziden Aktivität, das Serum der wilden Ratten den höchsten, dagegen jenes der Albinos den niedrigsten Properdinspiegel besitzt. Es scheint also zweifellos, dass der Properdinspiegel und jene Faktoren die beim Zustandekommen einer natürlichen Immunität eine Rolle spielen, nicht nur bei verschiedenen Arten, sondern auch bei Rassen derselben Art verschieden sind.

Die Kreuzung von wilden und Albinoratten zeigte interessante Ergebnisse. Der durchschnittliche Properdinspiegel der wilden Ratten ist 36.6 E/ml. Derselbe beträgt bei Albinos 18,3 E/ml und bei den Hybriden 48,3 E/ml. Hieraus geht hervor, dass die immunbiologischen Titer durch genetische Faktoren bestimmt werden und dass auch eine Heterosiswirkung möglich ist. Dies ist um so interessanter, als es in früheren Arbeiten gelang nachzuweisen, dass Umweltsfaktoren (wie z. B. Zucht bei niedriger Temperatur) den Grad der Immunität in kurzer Zeit verändern können. Der Grad der Immunität eines Lebewesens ist demnach sowohl von genetische, als auch von Umweltsfaktoren abhängig.

ВЛИЯНИЕ НАСЛЕДСТВЕННОСТИ И ОКРУЖАЮЩЕЙ СРЕДЫ НА ОБРАЗОВАНИЕ ЕСТЕСТВЕННОГО ИММУНИТЕТА КРЫС

Изучая наследственные отношения естественного иммунитета, автор показал, что иммуннобиологические титры, в частности титр комплемента сыворотки крови и ее бактерицидная активность, у различных видов крыс (серых, лабораторных белых и Вистар) различны. Самые высокие титры комплемента и бактерицидная активность наблюдаются у серых крыс, самые низкие — у белых лабораторных крыс. У гибридов, полученных при скрещивании Вистар и серых крыс, наблюдается промежуточная степень титра комплемента и бактерицидной активности. Автор также показал, что подобно титру комплемента и бактерицидной активности, наибольшее количество пропердина содержится также в сыворотке серых крыс, и наименьшее — в сыворотке белых лабораторных крыс. Таким образом, количество пропердина и факторы, играющие роль в образовании естественного иммунитета, отличаются не только у разных видов, но даже у различных подвидов.

Скрещивание серых и лабораторных белых крыс привело к неожиданным результатам. Содержание пропердина в сыворотке у серых крыс было 36,6 Е/мл, а в сыворотке лабораторных белых крыс 18,3 Е/мл; в то время сыворотка их гибридов содержала 48,3 Е/мл пропердина. Эти результаты говорят о том, что иммуннобиологические титры определены наследственными факторами и в них могут проявляться действия гетерозиса, несмотря на ранние исследования автора, несомненно показывавшие, что степень иммунитета за относительно короткое время может изменятся под влиянием окружающей среды (как например, суровые условия). Таким образом, степень иммунитета живого существа в одинаковой мере зависит как от наследственных факторов, так и от факторов окружающей среды.

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THE NUCLEAR APPARATUS OF REMANELLA MULTINUCLEATA KAHL (CILIATA, HOLOTRICHA)

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Synopsis

The nuclear apparatus of *Remanella multinucleata* KAHL (Ciliata, Holotricha, family Loxididae) consists of 7 to 35 macronuclei and of 2 to 16 micronuclei. Usually, about 20 macro- and 5 micronuclei are present. The micronuclei are rich in DNA and contain no RNA. The macronuclei contain several (up to 10) nucleoli of unequal size which are rich in RNA and proteins. The quantity of DNA per macronucleus is comparatively small which allows to consider these nuclei to be diploid. Periodically, the nucleolar apparatus of all macronuclei undergoes synchronous cyclical changes. The latter consist in a dissolution of the largest nucleolus of each macronucleus, thus forming a vacuole containing RNA and protein, and in a gradual displacement of the contents of this vacuole from the macronucleus to the cytoplasm. During division of the ciliate, all existing macro- and micronuclei are distributed at random between the daughter individuals. The macronuclei of this species, as well as those of other Loxodidae, are incapable of division. The reorganization of the nuclear apparatus takes place between two cell divisions, and includes transformation of a number of micronuclei into macronuclear anlagen. The number of macronuclei is thus supplemented. No sign of endomitotic polyploidization could be demonstrated during the development of the macronuclear anlagen.

Introduction

In a previous paper [11] we reported that in two species of the marine psammobiotic genus *Remanella* (family Loxodidae), namely in *R. rugosa* KAHL and *R. granulosa* KAHL, the macronuclei are unable to divide, poor in DNA, and apparently diploid. Both these forms possess 2 macronuclei and 1 micronucleus. During plasmotomy, the macronuclei are distributed without division, while the micronucleus divides mitotically twice. Each daughter individual receives 1 macronucleus and 2 micronuclei, one of the latter being gradually transformed into a macronucleus. This reorganizatory process thus results in restoration of the normal number of nuclei — 2 macronuclei and 1 micronucleus. A similar sequence of nuclear events was found in a fresh-water representative of the family Loxodidae, also having 2 macro- and 1 micronucleus, in *Loxodes rostrum* O. F. M. [8]. In a species with 2 macro- and 2 micronuclei — *Loxodes striatus* (ENGELM.) — changes of the nuclear apparatus during cell division are essentially analogous, but their sequence is somewhat different. Finally, in a third fresh-water species — the multinuclear *Loxodes magnus* STOKES — the mitoses of the micronuclei and the

transformation of a part of the micronuclei into macronuclei occur asynchronously between two divisions of the ciliate. During plasmatomy, only a random distribution of the macro- and micronuclei takes place in this species [8]. The diploidy of the macronuclei of *Loxodes magnus* was recently proved photometrically [13].

The macronuclei contain large nucleoli both in *Loxodes* and *Remanella*. Cyclical changes of the nucleoli related to a transfer of nucleolar RNA into the cytoplasm were observed [11] in *R. rugosa* and *R. granulosa*, while we failed to find such phenomena in *Loxodes*.

Keeping in mind these peculiarities of the nuclei of Loxodidae, we wished to study the caryological features of a multinuclear species of the genus *Remanella*, namely of *R. multinucleata* KAHL, and to compare its nuclear processes with those in other species of *Remanella* and *Loxodes*.

The structure of the nuclei of *R. multinucleata* was briefly described by FAURÉ-FREMIET [3] and DRAGESCO [1]; in particular, weak stainability of the macronuclei by the Feulgen reaction was noted. In another paper, FAURÉ-FREMIET [4] pointed out that the macronuclei of this species did not divide during plasmatomy, and suggested that the macronuclei of *R. multinucleata* as well as those of *Loxodes*, were incapable of division ("caryostérose"). The ciliature of *R. multinucleata* was thoroughly studied by DRAGESCO [2].

Material and methods

Specimens of *R. multinucleata* were collected in June—July, 1961, on an intertidal sandy beach of the White Sea, mainly on the Rjashkov Island within the Kandalaksha Reservation (for details see [10]). Table 1 shows a comparison of some taxonomic characters of *R. multinucleata* from the White Sea with data obtained from the literature.

Table 1

Some taxonomic characters of R. multinucleata

Character	KAHL [5, 6]	FAURÉ-FREMIET [3, 4]*	DRAGESCO [1, 2]	This study
Body length in microns	400—700	400—700	500—800	450—600
Number of cineties	?	?	20—22	25—30
Number of Müller's vesicles	2—5	4—8	4—6	2—10 (Mo=4)
Number of macronuclei	«zahlreiche«	~10—14	12—23	7—35 (Mo=20)
Number of micronuclei	?	~5—7	3—8	2—16 (Mo=5)

* This form is considered by DRAGESCO [1] to be a separate species, *R. faurei*.

The ciliates were fixed and attached to coverslips with Nissenbaum's [7] sublimate mixture. A part of the material was studied in 5μ sections after fixation with sublimateacetic acid mixture or Zenker's fluid. The preparations were stained with Heidenhain's iron hema-

toxyline, Mayer's hemalum or borax carmine. Cytochemical methods for nucleic acids (Feulgen reaction, methyl green – pyronin, toluidine blue and gallocyanin staining) were widely used. Proteins were stained with Mazia, Brewer and Alfert's mercuric bromphenol blue. In all, about 350 specimens of *R. multinucleata* were cytologically studied.

Results

Structure of the nuclear apparatus

The nuclear apparatus of *R. multinucleata* consists of 7 to 35 macronuclei and of 2 to 16 micronuclei, arranged in the middle part of the body in a longitudinal row (Fig. 1). For studying the variability of the number of nuclei, the macronuclei and the micronuclei were counted in 142 specimens of *R. multinucleata*. Histograms representing the distribution of these specimens according

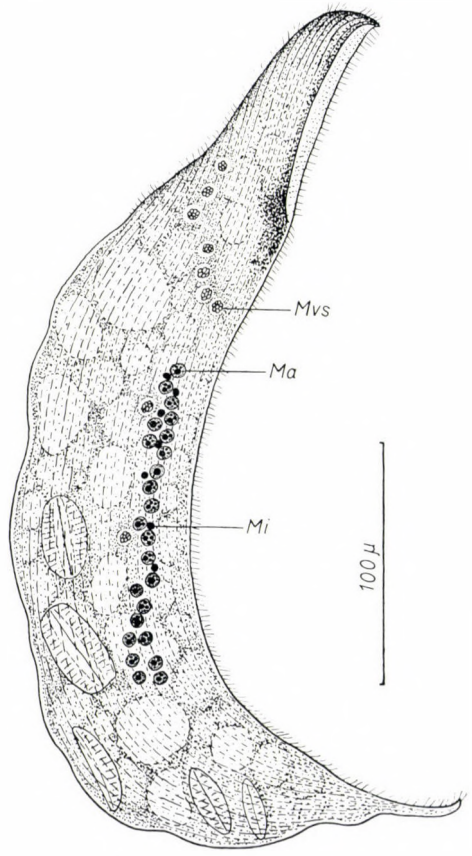


Fig. 1. Remanella multinucleata. Whole mount, stained with borax carmine; view from the right body face. *Ma* — macronucleus, *Mi* — micronucleus, *Mvs* — Müller's vesicles

to the numbers of the macro- and micronuclei are given in Fig. 2. The macronuclei as well as the micronuclei showed typical unimodal distribution curves indicating the homogeneity of the material. The modal number of macronuclei is 20, that of micronuclei is 5; the means (M $\pm$ m) are correspond-

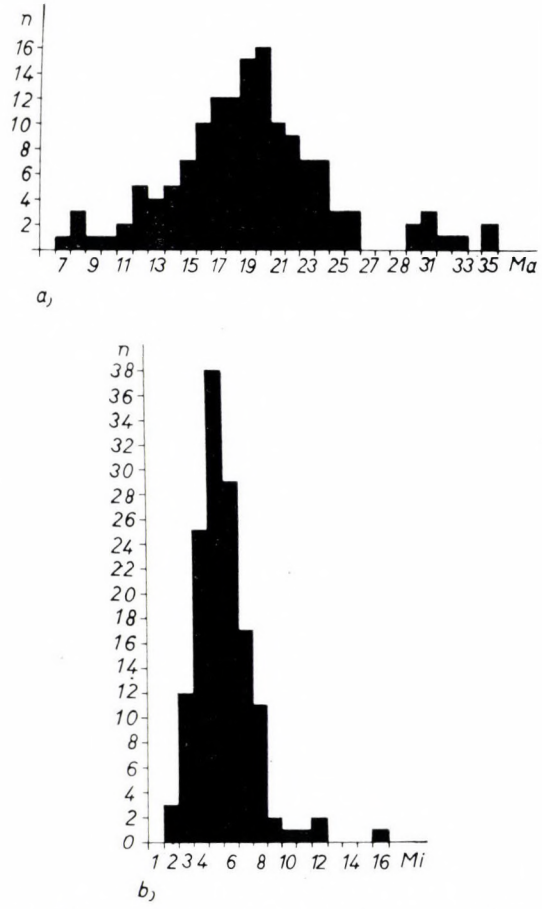
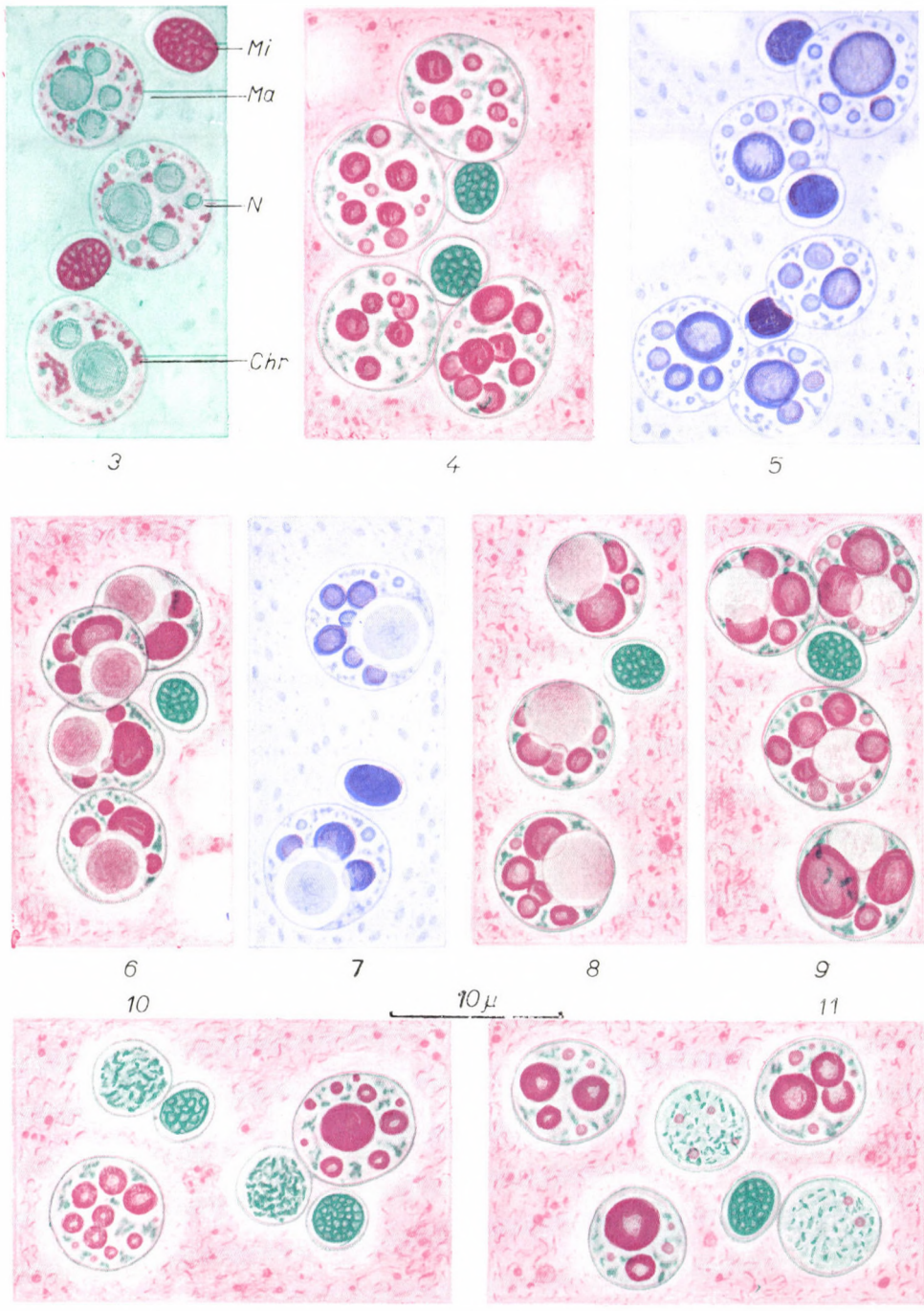


Fig. 2. Distribution of 142 specimens of *R. multinucleata* according to the numbers of macronuclei (*a*) and micronuclei (*b*). Abscissae: number of nuclei, ordinates: frequencies.

ingly 19.17 $\pm$ 0.42 and 5.58 $\pm$ 0.17. There is a definite positive correlation between the numbers of macro- and micronuclei (r = 0.592 $\pm$ 0.055).

The micronuclei of *R. multinucleata* are oval, 3.5 μ long. The chromatin of a micronucleus has a slightly vacuolar structure. It gives a strong Feulgen reaction (Fig. 3) and stains intensively with methyl green (Fig. 4), suggesting a high concentration of DNA and the absence of RNA. The micronuclei are rich in proteins, the reaction for total protein being clearly positive not only in the chromatin but also in the micronuclear membrane (Fig. 5).



Figs. 3 to *11*. Morphology and cytochemistry of nuclei of *R. multinucleata* (from whole mounts and sections). *Figs. 3* to *5* — macronuclei (with ordinary nucleoli) and micronuclei. *Figs. 6* to *9* — nucleolar cycle of the macronuclei: *Figs. 6* and *7* — formation of vacuoles around nucleoli; *Fig. 8* — dissolution of nucleoli, the stage of RNA-vacuoles; *Fig. 9* — the stage of clear vacuoles. *Figs. 10* and *11* — two stages of development of macronuclear anlagen. *Fig. 3* — Feulgen reaction, *Figs. 4*, *6* and *8* to *11* — methyl green — pyronin, *Figs. 5* and *7* — mercuric bromphenol blue. *Ma* — macronucleus, *Mi* — micronucleus, *N* — nucleolus, *Chr* — chromatin

The macronuclei are spherical, 6 to 9 μ in diameter; occassionally they have an oval or irregular shape (Figs. 3 to 5). The membrane of macronuclei gives definitely positive reactions for total protein only (Fig. 5); it does not contain RNA (Fig. 4). The DNA content of macronuclei is comparatively small, as it is in other species of *Remanella* and *Loxodes*. A moderate quantity of small granules and a few larger chromocenters in the karyolymph are the only Feulgen-positive elements of macronuclei (Fig. 3). Chromatin elements contain no RNA, staining pure green with methyl green — pyronin mixture (Fig. 4). Their protein content is relatively moderate (Fig. 5).

Macronuclei commonly contain several spherical nucleoli, the number of the latter attaining 10. The dimensions of the nucleoli in macronuclei are nearly always unlike: well visible larger nucleoli coexist with smaller ones, the smallest being at the limit of the resolving power of the light microscope. Sometimes the largest nucleoli do not exceed 2 μ in diameter (Fig 4), but more often one nucleolus stands out sharply among the others, being up to 3 μ or 4 μ large (Figs. 3 and 5). The nucleoli are Feulgen-negative (Fig. 3), definitely basophilic (particularly their periphery, Fig. 4). Control preparations digested with ribonuclease show that the stainability of nucleoli with pyronin, toluidine blue and gallocyanin depends or RNA. Nucleoli show also a high content of total proteins (Fig. 5).

Nucleolar cycle

Cyclical changes in the nucleolar apparatus, observed in the macronuclei of approximately 20 per cent of the examined specimens, are probably connected with a transfer of nucleolar RNA into the cytoplasm (Figs. 6 to 9). At the beginning of these changes, the largest nucleolus of each macronucleus becomes surrounded with a clear vacuole and is displaced to the periphery of the nucleus (Figs. 6 and 7). The content of RNA (Fig. 6) and proteins (Fig. 7) in this nucleolus diminishes so that, with adequate methods, it stains more faintly than the other nucleoli.

This nucleolus seems later to be dissolved in the contents of the surrounding vacuole. Distinct boundaries of the nucleolus fade away, and the vacuole appears to be filled with a moderately basophilic matter (Fig. 8). The strongest basophilia appears at the spot where the vacuole containing RNA and protein comes in contact with the nuclear membrane.

Then a gradual loss of basophilia occurs in the RNA-vacuole; simultaneously its protein content also diminishes. As a result the vacuole soon turns into a clear vesicle which gives practically negative reactions for RNA and protein (Fig. 9). The clear vacuole diminishes in size until it vanishes.

This phenomenon may be explained by postulating a transfer of RNA (and protein) from the vacuole of the macronucleus to the cytoplasm. No

emptying of the vacuole contents into the cytoplasm (as visible for example in *Geleia orbis* [12]) could be observed under the microscope in *R. multinucleata*. It is therefore possible that RNA migrates into the cytoplasm diffusely through submicroscopic pores of the nuclear membrane.

Nucleolar changes occur always synchronously in all macronuclei of a given specimen. The described cycle can apparently be repeated in a single macronucleus: one of the remaining nucleoli may grow and occupy the place of the dissolved one. After attaining a certain size, it may in its turn, give rise to an RNA-vacuole. We have so far failed to ascertain whether nucleolar processes in the macronuclei are temporally connected with the division of the ciliate.

Division

All present observations allow us to confirm the data of FAURÉ-FREMIET [4] about the absence of nuclear divisions during plasmotomy in *R. multinucleata*. During the division of the ciliate, the macro- and micronuclei are mechanically (and apparently at random) distributed between the two daughter individuals (Fig. 12). The daughter animals usually receive an unequal number of nuclei: so, in Fig. 12, the anterior cell receives 12 macronuclei and 3 micronuclei, the posterior one 13 macronuclei and 5 micronuclei.

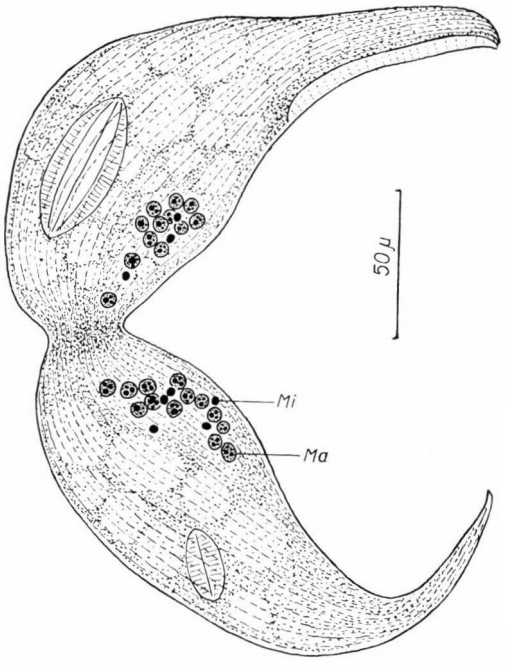


Fig. 12. Division of *R. multinucleata*. Whole mount, hemalum stained, viewed from the right. *Ma* — macronucleus, *Mi* — micronucleus

The reorganization of the nuclear apparatus, involving an increase in the number of nuclei, occurs during the interdivision period (as in *Loxodes magnus* [8]). The micronuclei apparently multiply by means of mitoses; yet in spite of a considerable number of specimens studied, we failed to find any figures of micronuclear division. The mitoses are presumably of a very short duration. A number of micronuclei transform into macronuclear anlagen between two cell divisions, as well. The formation of macronuclear anlagen is more or less synchronous in *R. multinucleata* : the anlagen which were observed in approximately 10 per cent of the individuals were always present in considerable numbers (5—10) and at identical stages of development (Figs. 10 and 11).

The beginning of the development of macronuclear anlagen (Fig. 10) resembles a mitotic prophase. A knotty net-like chromatin structure, obviously formed by chromosomes, is visible within the anlagen. The diameter of an anlage is at this stage approximately 5 μ. The chromatin yields negative reactions for RNA, staining pure green with the methyl green—pyronin mixture (Fig. 10). The macronuclear anlagen grow subsequently to 6—7 μ, and several small nucleoli containing RNA appear therein (Fig. 11). Chromatin elements become more loose and pale, probably as a result of chromosome despiralization. No signs of duplication of chromatin elements or of DNA synthesis to indicate an endomitotic process were found in the macronuclear anlagen.

Discussion

A comparison of the nuclear apparatus of *Remanella multinucleata* with that of other lower ciliates (members of the family Loxodidae in particular) reveals a number of interesting peculiarities. First, the macronuclei of *R. multinucleata* should be regarded as diploid like the macronuclei of many other lower ciliates. This feature distinguishes them sharply from the macronuclei of higher ciliates which are known to be highly polyploid (for references see [13]). The conclusion regarding the diploidy of the macronuclei in *R. multinucleata* is based on their low chromatin (DNA) content and on the absence of any sign of endomitosis during the development of macronuclear anlagen.

The sequence of nuclear phenomena seems to depend directly on the number of nuclei. In all forms with a small and constant number of macro- and micronuclei, mitoses of micronuclei and the development of macronuclear anlagen occur synchronously during plasmotomy (*Loxodes rostrum*, *L. striatus*, *Remanella rugosa*, *R. granulosa*, *Geleia nigriceps*, *G. orbis* etc. [8, 9, 11, 12]). Nuclear reorganization occurs during the interval between two divisions in all multinuclear forms. In *Loxodes magnus*, the mitoses of the micronuclei and the formation of the macronuclear anlagen are asynchronous. In *Rema-*

nella multinucleata, these processes are apparently more synchronized although they do not take place during plasmotomy. All multinuclear forms with diploid macronuclei are characterized by a wide variability of the number of nuclei, a phenomenon due to the random distribution of nuclei during plasmotomy.

As to the nucleolar cycle, it certainly reflects a high physiological activity of diploid somatic nuclei — the macronuclei. At present, most investigators believe that all RNA of the cell or at least a major part of it is synthesized within the nucleus, and migrates thereafter into the cytoplasm. One of the RNA fractions, the so-called messenger RNA, was shown to carry genetic information from the nuclear chromosomes to the centers of protein synthesis in the cytoplasm. In this connection, data about transfer of RNA from the macronuclear nucleoli of *R. multinucleata* to the cytoplasm are, worthy of note.

As regards the character of the nucleolar cycle, *R. multinucleata* resembles most closely *Remanella granulosa* [11]. In both species only a single RNA vacuole is formed in each macronucleus, and the "secretion" of RNA into the cytoplasm occurs diffusely, without rupture of the nuclear membrane. In *R. rugosa* all nucleoli of the macronucleus dissolve simultaneously [11], while species of *Geleia* are characterized by emptying the contents of RNA vacuoles into the cytoplasm through a gap in the nuclear membrane *(Geleia orbis* [12]), or by extrusion from the macronucleus of a dense RNA sphaerulum, likewise through a rupture of the nuclear membrane *(Geleia nigriceps* [9]).

The presence or absence of a nucleolar cycle does not appear to depend on the number of nuclei. This cycle is characteristic of all species of *Remanella* whether they have two or more macronuclei, and also of all members of the genus *Geleia* [9, 12]. On the other hand, in *Loxodes* and in Trachelocercidae, we failed to find any microscopic nucleolar changes connected with RNA migration into the cytoplasm.

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DER KERNAPPARAT VON REMANELLA MULTINUCLEATA KAHL (CILIATA, HOLOTRICHA)

Der Kernapparat von *Remanella multinucleata* Kahl (Ciliata, Holotricha, Familie Loxodidae) besteht aus 7 bis 35 Makronuclei und 2 bis 16 Mikronuclei. Gewöhnlich sind ungefähr 20 Makronuclei und 5 Mikronuclei vorhanden. Die Mikronuclei sind reich an DNS und enthalten keine RNS. Die Makronuclei enthalten mehrere (bis 10) Nucleolen ungleicher Grösse, die an RNS und Proteine reich sind. Die Menge von DNS in einem Makronucleus ist verhältnissmässig klein so, dass diese Kerne als diploid betrachtet werden können. Periodisch wird der Nucleolarapparat aller Makronuclei synchronen zyklischen Veränderungen unterworfen. Die Letzteren bestehen in der Auflösung des grössten Nucleolus jedes Makronucleus, wobei eine RNS- und proteinhaltige Vakuole gebildet wird, und in einer allmählichen Abgabe des Inhaltes dieser Vakuole aus dem Makronucleus ins Cytoplasma. Während der Teilung des Ciliats geschieht eine zufällige Verteilung aller vorhandenen Makro- und Mikronuclei zwischen den Tochtertieren. Die Makronuclei dieser Art, wie auch solche anderer *Loxodidae*, sind gänzlich teilungsunfähig. Die Reorganisation des Kernapparates findet zwischen zwei Teilungen statt; sie besteht in einer Umwandlung eines Teiles der Mikronuclei in Makronucleusanlagen. Dadurch wird die Makronucleuszahl ergänzt. Während der Entwicklung der Makronucleusanlagen fehlen jegliche Anzeichen einer endomitotischen Polyploidisierung.

ЯДЕРНЫЙ АППАРАТ REMANELLA MULTINUCLEATA KAHL (CILIATA HOLOTRICHA)

Ядерный аппарат *Remanella multinucleata* Kahl (Ciliata, Holotricha, сем. Loxodidea) состоит из 7—35 макронуклеусов и 2—16 микронуклеусов. Чаще имеется около 20 макро- и 5 микронуклеусов. Микронуклеусы богаты ДНК и не содержат РНК. Макронуклеусы содержат несколько (до 10) нуклеол неодинакового размера, богатых РНК и белками. Количество ДНК в одном макронуклеусе сравнительно невелико, что позволяет считать эти ядра диплоидными. Периодически нуклеолярный аппарат всех макронуклеусов подвергается синхронным циклическим изменениям. Последние заключаются в растворении самой крупной нуклеолы каждого макронуклеуса, превращении ее в вакуоль, содержащую РНК и белок, и в постепенном выходе содержимого вакуоли из макронуклеуса в цитоплазму. Во время деления инфузории происходит случайное распределение имеющихся в наличии макро- и микронуклеусов между дочерними особями. Макронуклеусы этого вида, как и других Loxodidae вообще неспособны к делению. Реорганизация ядерного аппарата происходит в промежутке между двумя делениями и включает превращение части микронуклеусов в зачатки макронуклеусов. Тем самым число последних пополняется. При развитии зачатков макронуклеусов отсутствуют какие-либо признаки эндомитотической полиплоидизации.

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ELECTRON MICROSCOPE STUDIES ON NEUROMUSCULAR JUNCTIONS OF END-PLATE TYPE IN INSECTS

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Synopsis

Ultrastructural organization of an "end-plate" type of insect fibered(leg-muscle innervation is described in *Apis mellifica* (Hymenoptera) and *Geotrupes vernalis*, *Cetonia aurata* (Coleoptera). This type appears to be common in the fibered(leg-) muscles of Hymenoptera and Coleoptera and is one of the simplest end-plate types of insect muscle innervation. Effective contact between axon terminal and muscle fiber is established by pillar-like plasmatic protrusions of the latter that are attached from both sides alternatingly to small regions of the axon. All other parts of the axon terminal are covered by a thin cellular lamina of the lemmoblasts and separated from the muscular (plasma) membrane by a complicated labyrinth of intercellular cysterns. Some prominent structural properties of lemmoblastic cellular elements, axon terminals and particularly of postsynaptic muscle fiber regions are described and their possible functional significance briefly referred to.

Introduction

Neuromuscular junctions of insects have widely been investigated with the aid of light microscopy [1, 7, 12, 13, 14] and recently also by the electron microscope [2, 3, 4, 16]. According to all information available, insect muscle innervation is "multiterminal" — i. e. each muscle fiber having several nerve endings or contacts with nerve elements — and in many cases "polyneural", i. e. the same muscle fiber and/or ending being supplied by nerve fibres of different origin. A good example of this type of innervation is the highly specialized jumping muscle of the locusts, which, according to the investigations of HOYLE [9] is innervated at the same sites by "fast" as well as "slow" fibres. From many confusing and often seemingly controversial data on morphological and functional properties scattered in rather haphazard manner over different orders of insects and also over different muscles of the same species it is obvious that a systematic analysis of the structure of neuromuscular junction in insects is badly needed before any attempt can be made to correlate histological and functional properties on a broader scale. We have been trying [8] to reduce the wealth of structural details to a few main types of neuromuscular junction, with the overall architectural design being identical or at least similar. Apart from the difference already mentioned that the innervation may be "mononeural" — albeit "multiterminal" — or "poly-

neural" — two entirely different types of arrangement of neuromuscular junction can readily be recognized even at the level of the light microscope: neuromuscular endings of (1) the "endplate" type and (2) of a generally more diffuse arrangement which for the sake of simplicity might be called "non end-plate" type. The former type appears to be more general in the fibered, (leg-)muscles of Coleoptera and Hymenoptera, but by no means obligatory, since more diffuse types of innervation occur in the leg muscles of certain Coleoptera as for example *Dytiscus* [5]. The latter is the main type of neuromuscular junction in the fibrillar (flight-)muscle of Coleoptera and Hymenoptera. The leg muscles of Orthoptera have a rather complex mode of innervation with diffuse and end-plate types combined [7].

As there is not much to be gained by an attempt to establish a morphological system of insect muscle innervation merely on the level of the light microscope, we have started a comparative series of electron microscope studies of this subject, with the aim to bring some order and understanding at the ultrastructural level into the host of controversial or not sufficiently related structural details. Such investigations are expected to supply sooner or later a sounder basis for future systematization. The present paper is the first of a series dealing exclusively with a comparatively simple form of "end-plate" type innervation.

Material and methods

Femoral muscles of *Cetonia aurata* and *Geotrupes vernalis* (Coleoptera) as well as of *Apis mellifica* (Hymenoptera) were prefixed in situ for a few minutes with 1 per cent OsO_4 solution buffered to pH 7,5, then dissected and the fixation continued for 1 hour at 4° C in the same fixative. The muscles were then dehydrated in graded ethanol, stained in some cases with 1 per cent phosphotungstic acid in absolute ethanol for 1 hour and embedded in Araldite [11]. Ultrathin sections were cut with the LKB-Ultrotome, floated on distilled water: from there they were picked up on copper grids covered with Formvar films. Some sections were stained with lead hydroxyde [10], others left untreated. Observations were made with the Zeiss (Jena) El-Mi. II, and "Tesla" electron microscopes.

Results

A detailed description of peripheric nerves being beyond the scope of this paper, only two pictures are given in Figs 1 and 2 of the femoral nerve in the honey bee (*Apis mellifica* with two large and one (or in other cases several) small axons, while in *Cetonia aurata* there occur generally but one large axon and several smaller ones additionally. The axons are ensheathed by lemmoblasts, highly different, however, in structure from those of the vertebrates. Instead of the clear relation between lemmoblast and unmyelinated fibre of the vertebrate with its mesaxon, a complex system of only more or less circular double membranes is seen to surround the larger axons in a pattern resembling the "loose myelin" structures of some sensory [15] and most

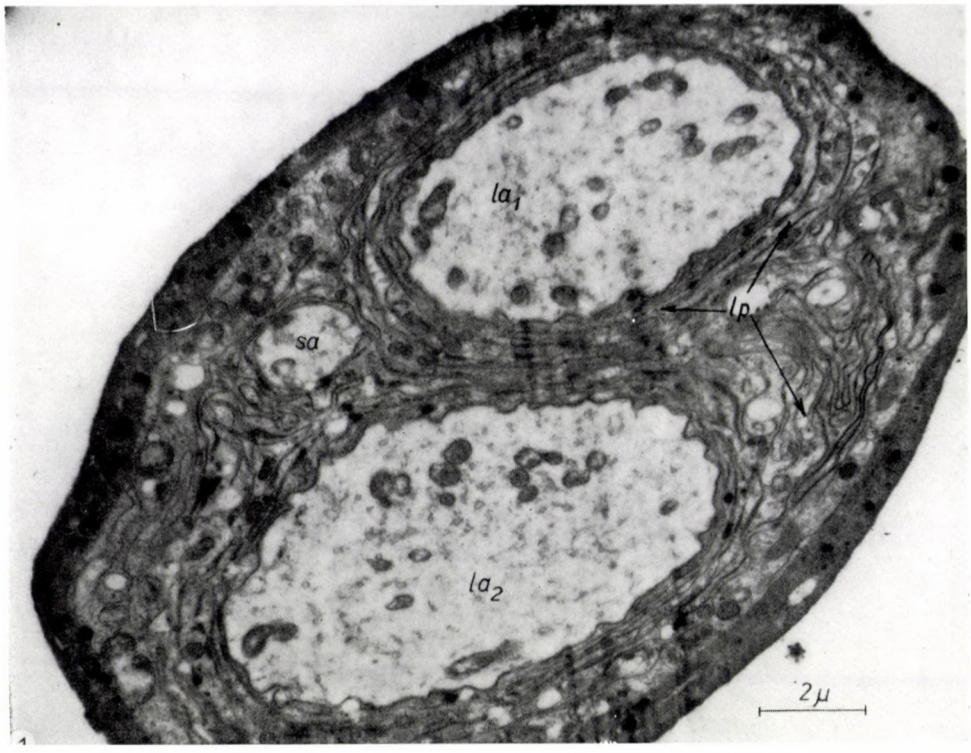


Fig. 1 Cross section of peripheral (leg) nerve in *Apis mellifica*. Two large (la_1, la_2) and one smaller (*sa*) axons covered by several layers of thin lemmoblast processes (*lp*). No mesaxons may be seen in this section. × 8000

autonomic ganglion cells (own unpublished observation). True mesaxons, however, can be recognized in groups of smaller axons, the relation of which to the lemmoblasts appears to be entirely similar to that of preterminal autonomic fibres to the Schwann cell (Fig. 2); The nucleus of the lemmoblast is situated on the periphery (Fig. 2); so are some axon-like profiles containing strange osmiophilic bodies, which can be seen especially in *Geotrupes* below the basement membrane embedded into the surface of the lemmoblast. We shall return to these profiles and their osmiophilic granules in the description of the terminals. The expression "tunicated" [2] is therefore appropriate also for the distinction from the myelinated fibre of the vertebrate. This expression should not, however, obscure the fact, that we do not know the real relation between the lemmoblasts and the larger axons, whether it is essentially a loose circular, albeit irregular, arrangement of a true mesaxon with a plasmatic cellular layer containing mitochondria (Fig. 1) in between two double membranes, or whether the plasma of the lemmoblast is simply longitudinally folded in an intricate manner and the folds of several lemmoblasts are interdigitating,

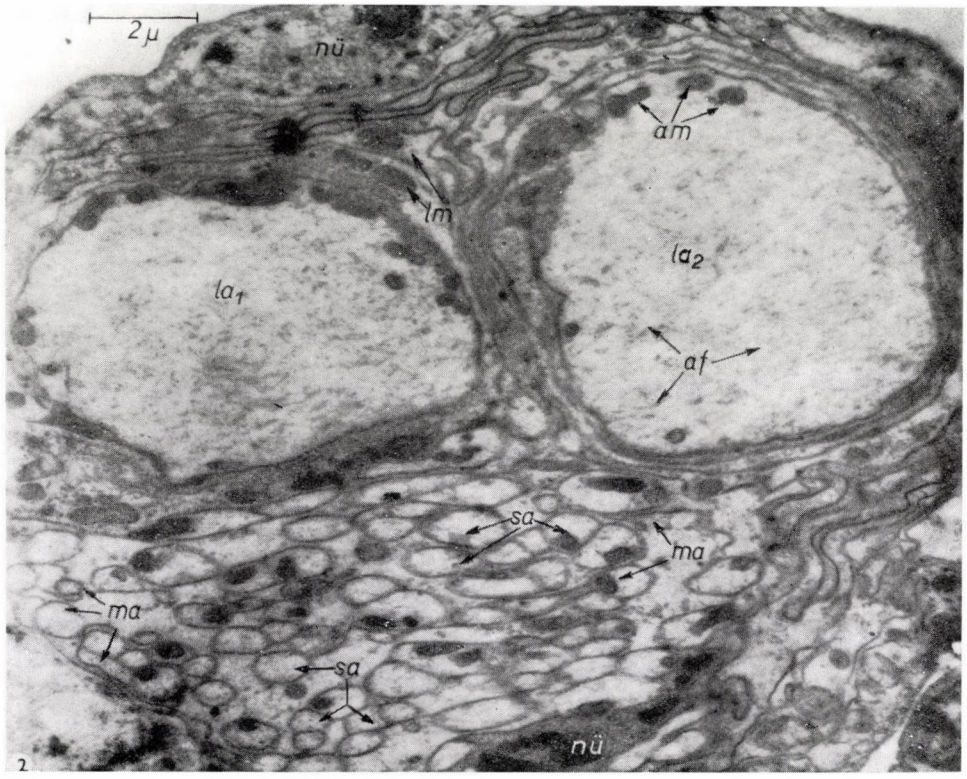


Fig. 2. Cross section of peripheral (leg) nerve in *Apis mellifica*. Two large and several smaller axons; only the smaller axons have mesaxons (*ma*). The nucleus of the lemmoblast (*nu*) occurs at the periphery of the nerve. Axons are filled with axofilaments (*af*); both axons and lemmoblasts contain mitochondria (*am*, *lm*). × 8500

with the larger axons wedged in between, as schematically indicated in Fig. 3. The relation of the smaller axons to the lemmoblast is shown at the cross section of the lower cell and, as mentioned, does not differ from that in the vertebrate small unmyelinated fibre. An outer layer of the lemmoblasts with significantly denser plasmatic structure, forms some kind of "basement membrane" on the surface of the nerve. More detailed investigations involving probably plastic reconstruction of ultrathin section series as well as experimental degeneration, will be, however, necessary in order to clarify the structure of peripheric insect nerves.

The neuromuscular end-plate in its simplest form — more complex types will be described in a forthcoming paper — is essentially a longitudinal attachment of an undivided terminal fibre to a specialized and somewhat elevated part of the muscle fibre (Doyère's hillock). The lemmoblastic tunic of the preterminal fibre withdraws from the site of attachment and covers the end-plate region with the bulk of its plasmatic body from the outer surface.

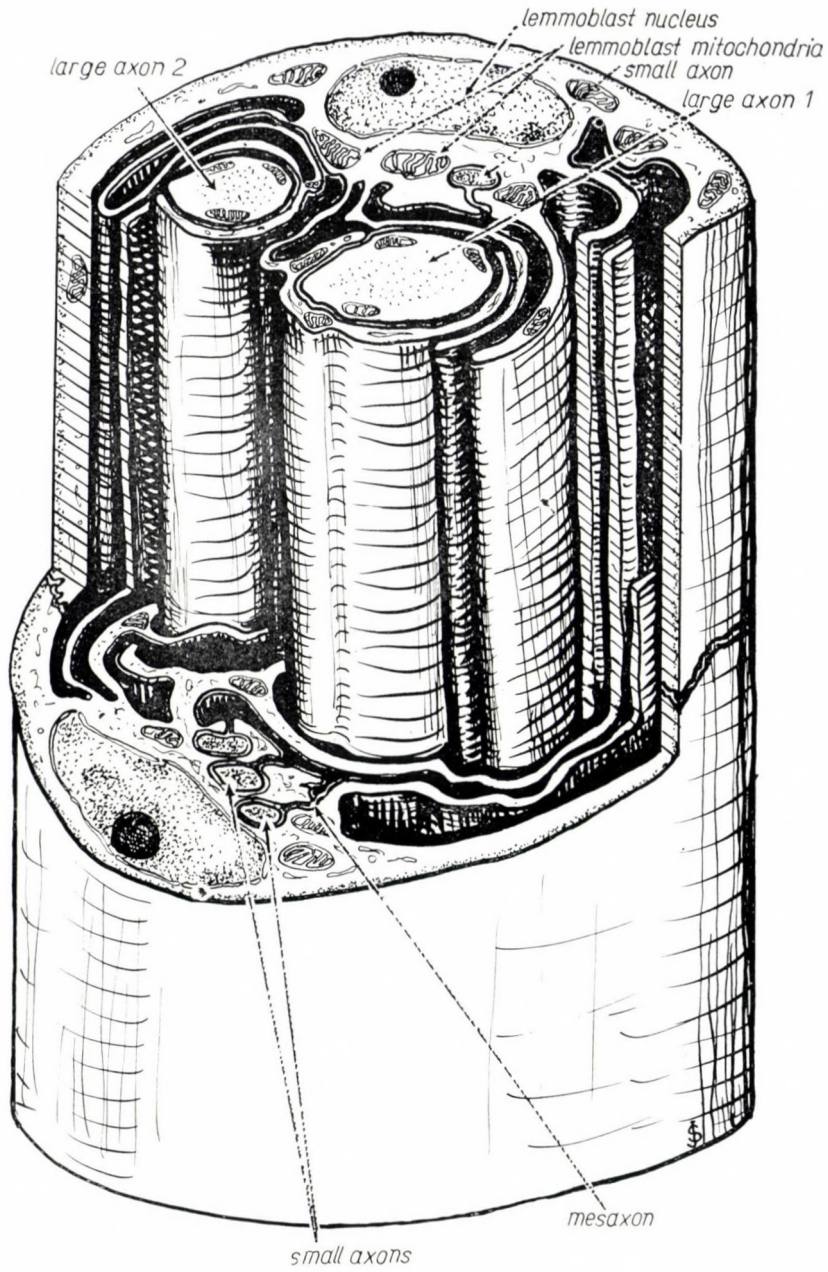


Fig. 3. An attempt to explain the structure of the peripheral nerve of *Apis mellifica*, *Cetonia aurata*, and *Geotrupes vernalis*, in a highly schematized stereoscopic diagram showing the relationship between large and smaller axons and lemmoblasts. Only smaller axons have mesaxon

The elevated muscular part of the end-plate consists of alternating, somewhat curved sarcoplasmatic pillars, which protrude from both sides and attach themselves closely with their concave surfaces — concavities looking inward — to both sides of the terminal part of the axon, as shown in a stereoscopic diagram (Fig. 4). All other parts of the axonal surface, especially the side facing the muscle fibre, are explicitly separated from the latter. These structural proper-

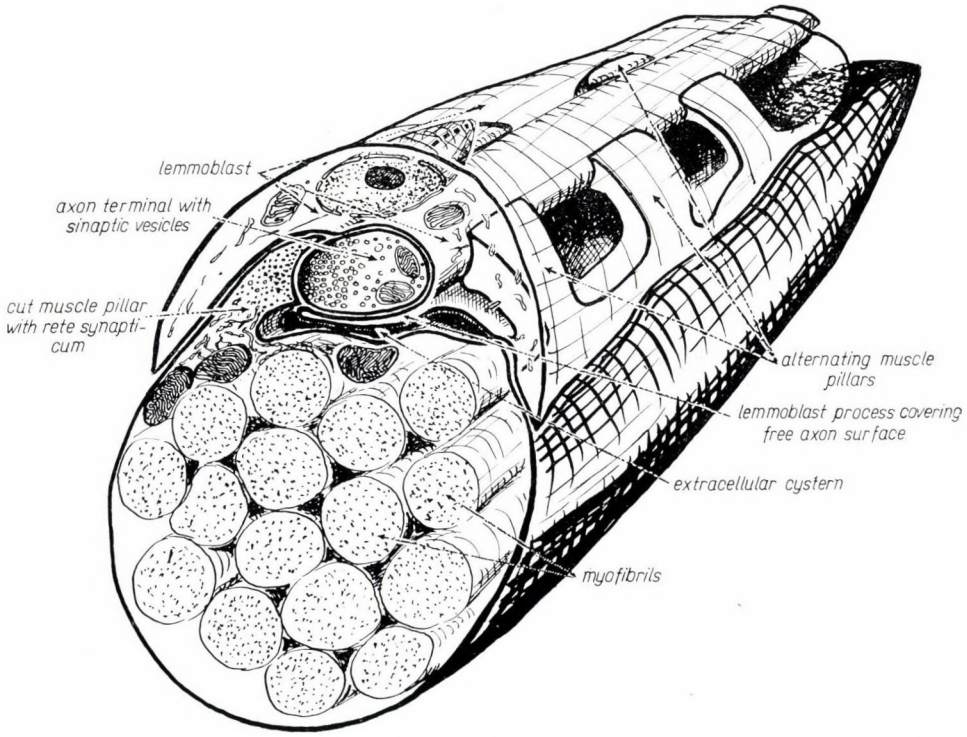


Fig. 4. Stereoscopic diagram of end-plate in *Apis mellifica*, *Cetonia aurata*, and *Geotrupes vernalis*, showing relationship between axon terminal, muscle cell, muscle pillars and lemmoblast. Extracellular cystern around pillars and particularly between the nerve fibre and the surface of the muscle cell situated between the origin of the pillars is also illustrated

ties can be seen clearly in Fig. 5, showing a slightly oblique cross section — hence two seemingly opposite pillars in the same plane — of an end-plate with a single large axon terminal, filled with synaptic vesicles of the usual size, as seen in most synapses of vertebrates. The inset photomicrograph (Fig. 6) of higher magnification shows the dense structure of the postsynaptic pillar with its clearly thickened postsynaptic membrane. Concerning the more detailed structure of the postsynaptic membrane nothing can be inferred from pictures of such resolving power. — Often it appears as if the dense postsynaptic pillars were separated from the muscle fibre by membrane

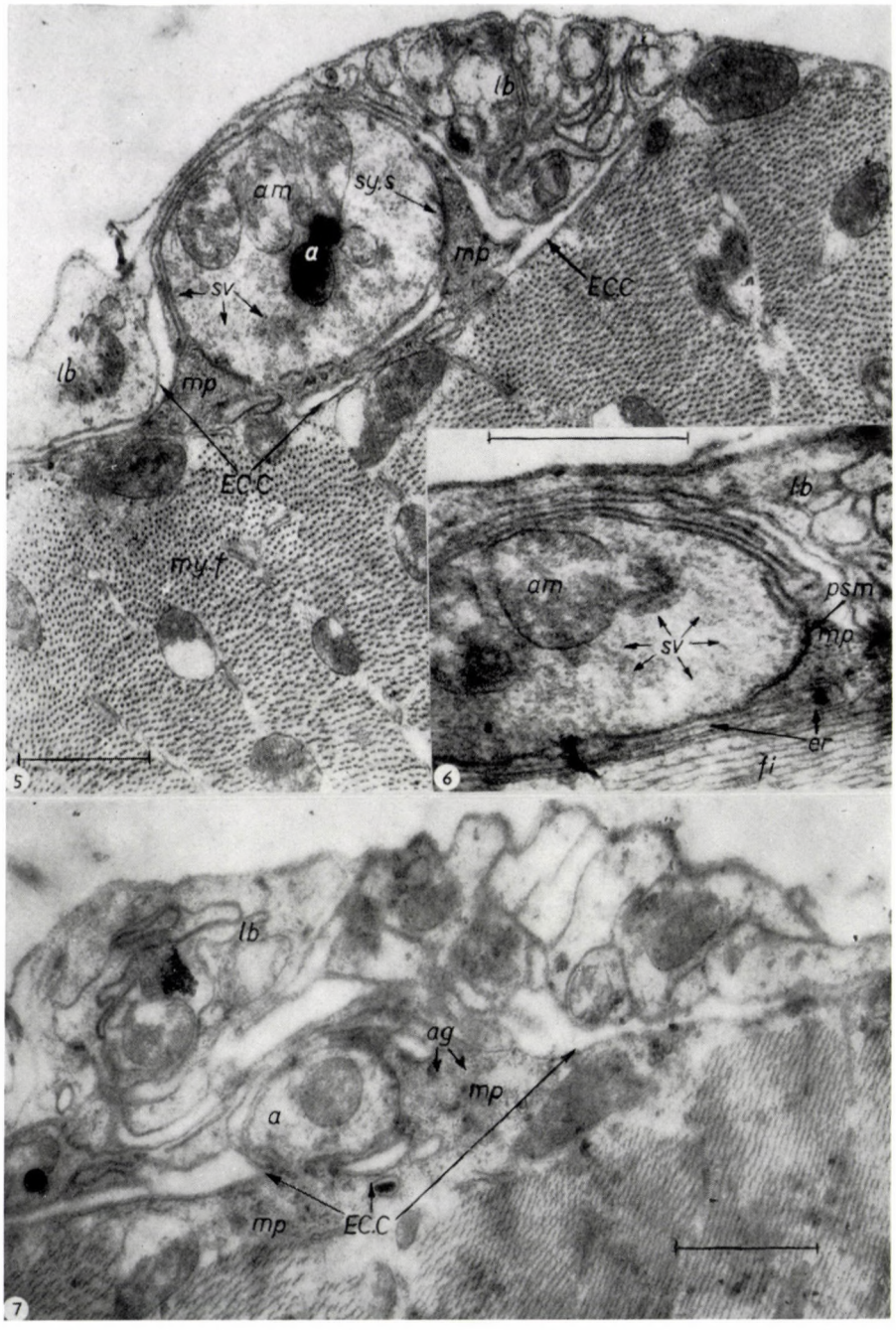
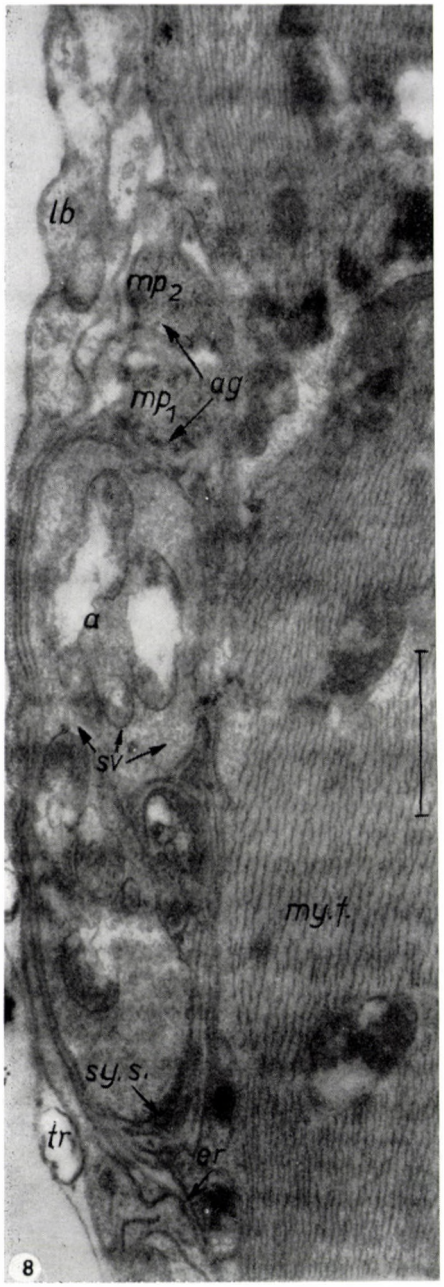


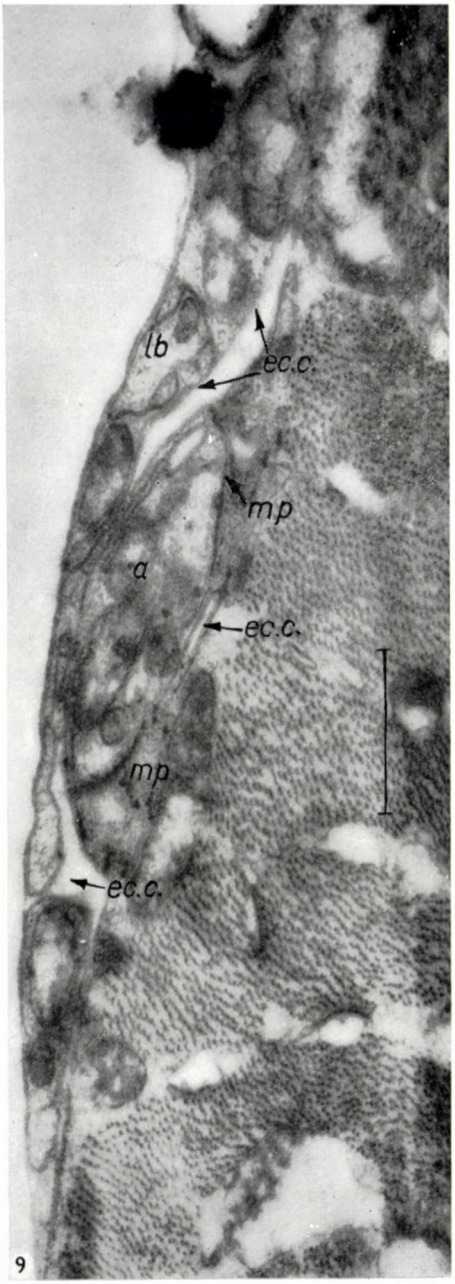
Fig. 5. Semi-cross section of an end-plate showing axon terminal (*a*), muscle pillars (*mp*), lemmoblast (*lb*) and extracellular cystern (*ec. c*). *am* — axon mitochondria; *my. f* — myofilaments; *sv* — synaptic vesicles. *Apis mellifica*, × 21,000. *Fig. 6.* Higher power electron micrograph, illustrating the apparent localized thickening of the postsynaptic membrane (*ps. m*) at the region of synapse. Note postsynaptic endoplasmic reticulum (*er*) connecting the base of muscle pillar (*mp*) with the surface of myofibril (*fi*). *Apis mellifica*, × 32,000. *Fig. 7.* Oblique section of an end-plate with an extended lemmoblast envelope (*lb*) and extracellular cystern (*ec. c*). *Apis mellifica*, × 20,000

systems, generally tangential to the circumference of the muscle fibre (Fig. 5). This is, however, only caused by a system of membranes suggestive of an endoplasmic reticulum closely connected with the bases of the pillars (Figs 6 and 8). It is difficult to trace the further connections of this membrane system — corresponding to *rete synapticum* of EDWARDS [3] — in this type of neuromuscular junction. It is much more prominent in other types of neuromuscular endings and will therefore be treated in more detail in subsequent papers.

The relation of the lemmoblasts to the neuromuscular junction can be seen much better in semilongitudinal (Fig. 10), or longitudinal (Fig. 12) sections. As seen in Fig. 10, the lemmoblast covers the whole surface of the axon, that is left free from attachment to the postsynaptic pillars with a thin plasmal layer having strange finger shaped villous protrusions, which hang freely into a system of extracellular cysterns of the end-plate. The extracellular cystern system surrounds most part of the terminal axon and separates it also from the muscular part of the ending, with the exception, of course, of the sites where the pillars are attached. As seen in several of the figures (Figs 5, 7, 9, 10 bottom) this cystern system communicates with the free intercellular space either through the clefts between muscle fibre and lemmoblasts or through clefts separating the cellular areas of different lemmoblasts. — Most part of the postsynaptic pillars is free of attachment of lemmoblast processes so that they are separated from the intercellular cysterns only by the basement membrane. As seen in Figs 8 and especially 12, tracheoles invade the region of the end-plate even as far as between axon and muscle fibre.

Very strange findings are profiles, somewhat resembling axon transverse sections, mentioned already in the femoral nerves, which contain very large osmiophilic bodies (Fig. 11). They are clearly separated from the cell body by a double membrane, one being the invaginated surface membrane of the lemmoblast, the other their own limiting membrane. They have never been observed in immediate connexion with the muscle fibre, but have always a rather large free surface on the outer side of the end-plate, covered only by a thin basement membrane. Our original explanation was that they must be some kind of neurosecretory nerve endings. This inference was based (1) on the analogy of the so called neurosecretory type synapses with large osmiophilic granula or vesicles as frequently seen in vertebrates, (2) on the occurence in these profiles of vesicular structures resembling synaptic vesicles, and (3) on observation of similar profiles in the peripheric nerves leading to these endings. They are very rare in *Apis*, but common in the end-plates of *Geotrupes*. Now we are not sure of our explanation mainly because we could never observe such profiles in longitudinal section. It might therefore well be, that these profiles correspond to vesicular inclusions of the lemmoblasts of unknown nature (neurosecretory? pathological-virus-?).





Figs. 8 and *9*. Oblique sections of end-plates. *Fig. 8*. Two neighbouring pillars (mp_1, mp_2) may be seen, which came into the same section because of its highly oblique plane. *a* — axon; *ag* — aposynaptic granules; *er* — endoplasmic reticulum; *lb* — lemmoblast; *my. f* — myofilaments; *sy. s* — synaptic surface; *sy* — synaptic vesicles; *tr* — tracheoles. *Apis mellifica*, × 21,000. *Fig. 9*. Two opposite pillars in close contact with the axon terminal. See relationship of extracellular cystern (*ec. c*) to axon (*a*), lemmoblast (*lb*) and muscle fibre. *Geotrupes vernalis*, × 21,000

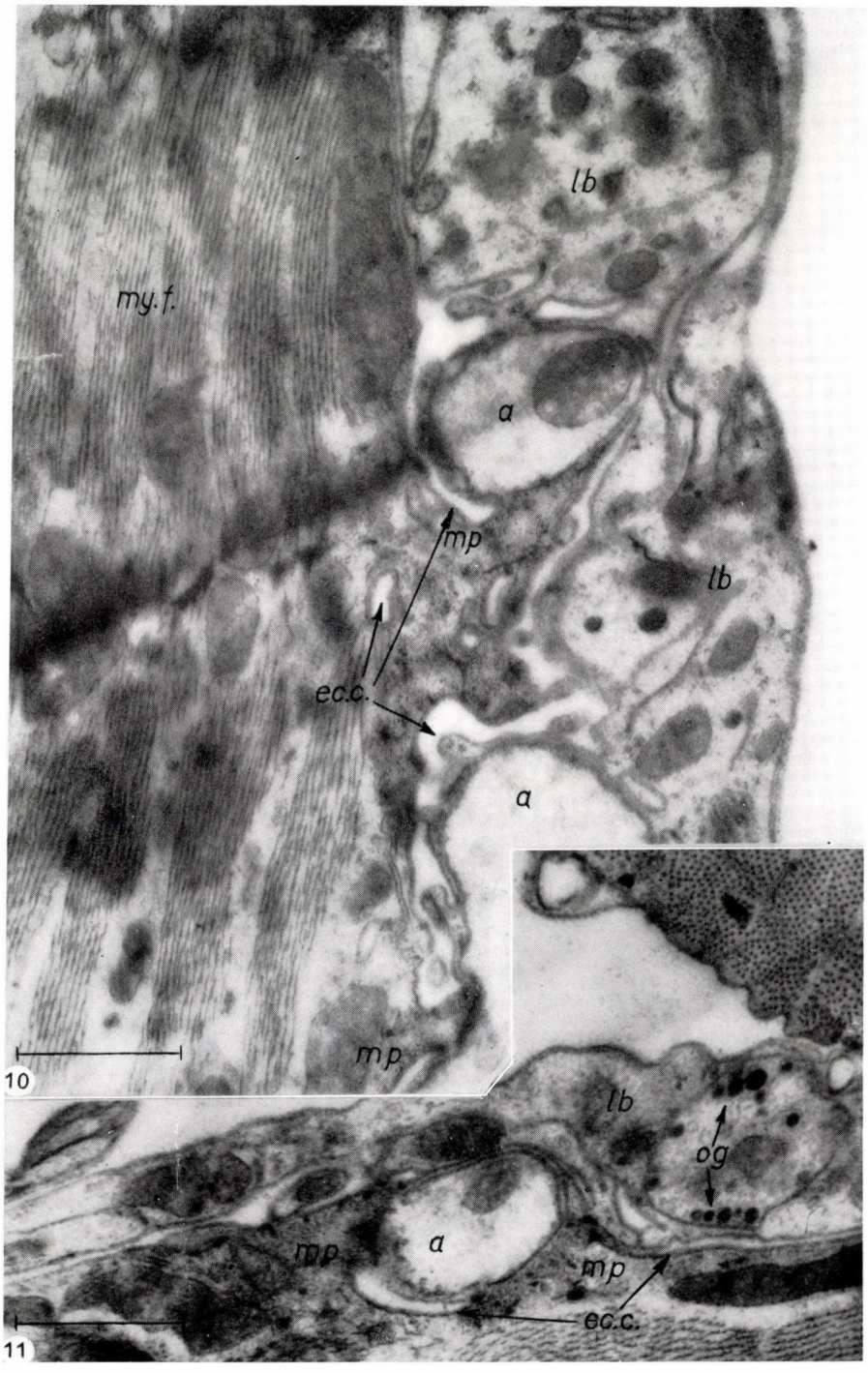


Fig. 10. Semilongitudinal section of end-plate. *a* — axon; *ec. c* — extracellular cystern; *lb* — lemmoblast; *mp* — muscle pillars; *my. f* — myofilaments. *Apis mellifica*, × 21,000.
Fig. 11. Oblique section of end-plate with osmiophilic granules (*og*) embedded into the lemmoblast (*lb*). *Apis mellifica*, × 21,000

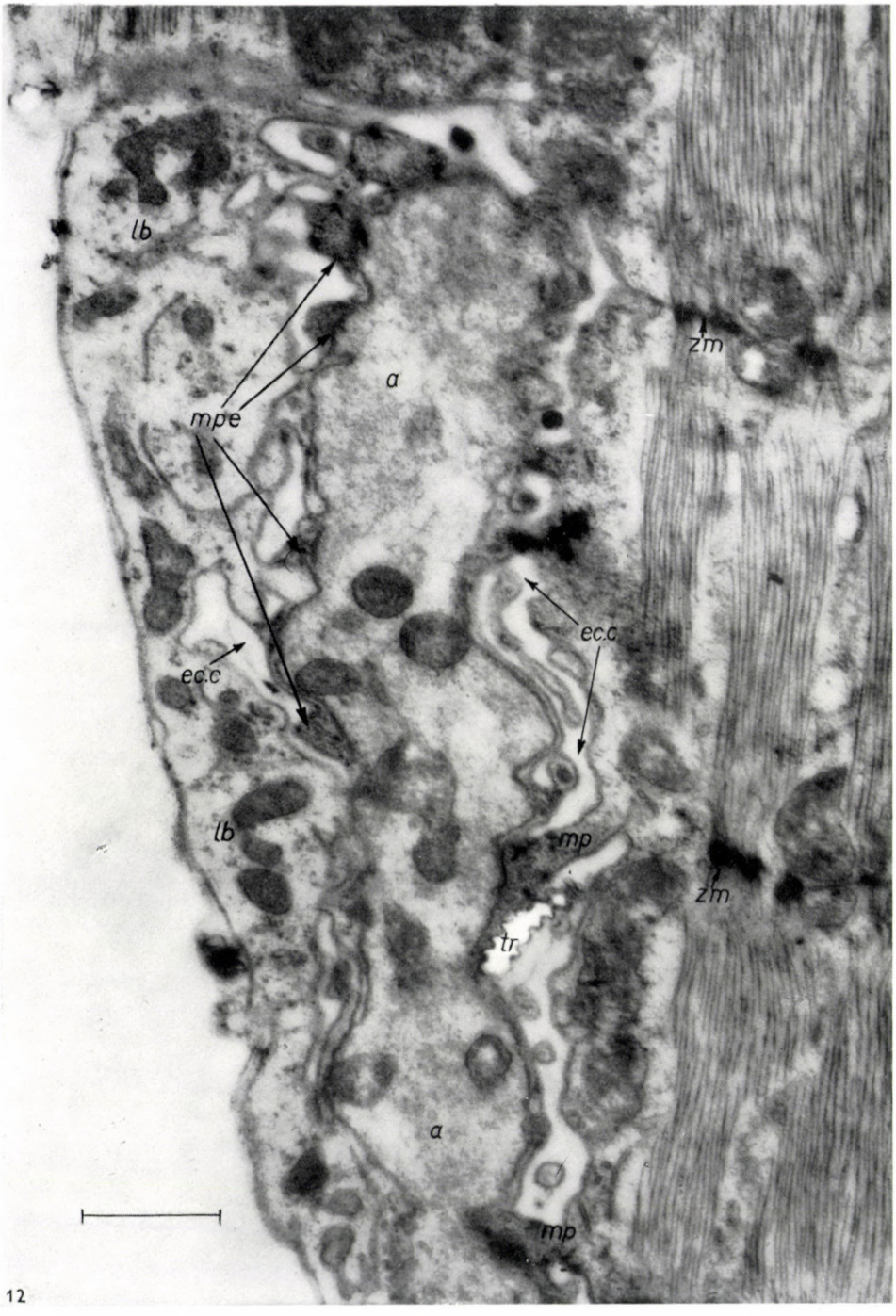


Fig. 12. Longitudinal, somewhat tangential section of an end-plate, showing on one side the pillars (*mp*), the surrounding cystern (*ec. c*) and on the opposite side some of the ends of the alternating opposite pillars (*mpe*). *a* — axon— *lb* — lemmoblast; *tr* — tracheole; *zm* — Z-membrane. *Apis mellifica*, × 21,000

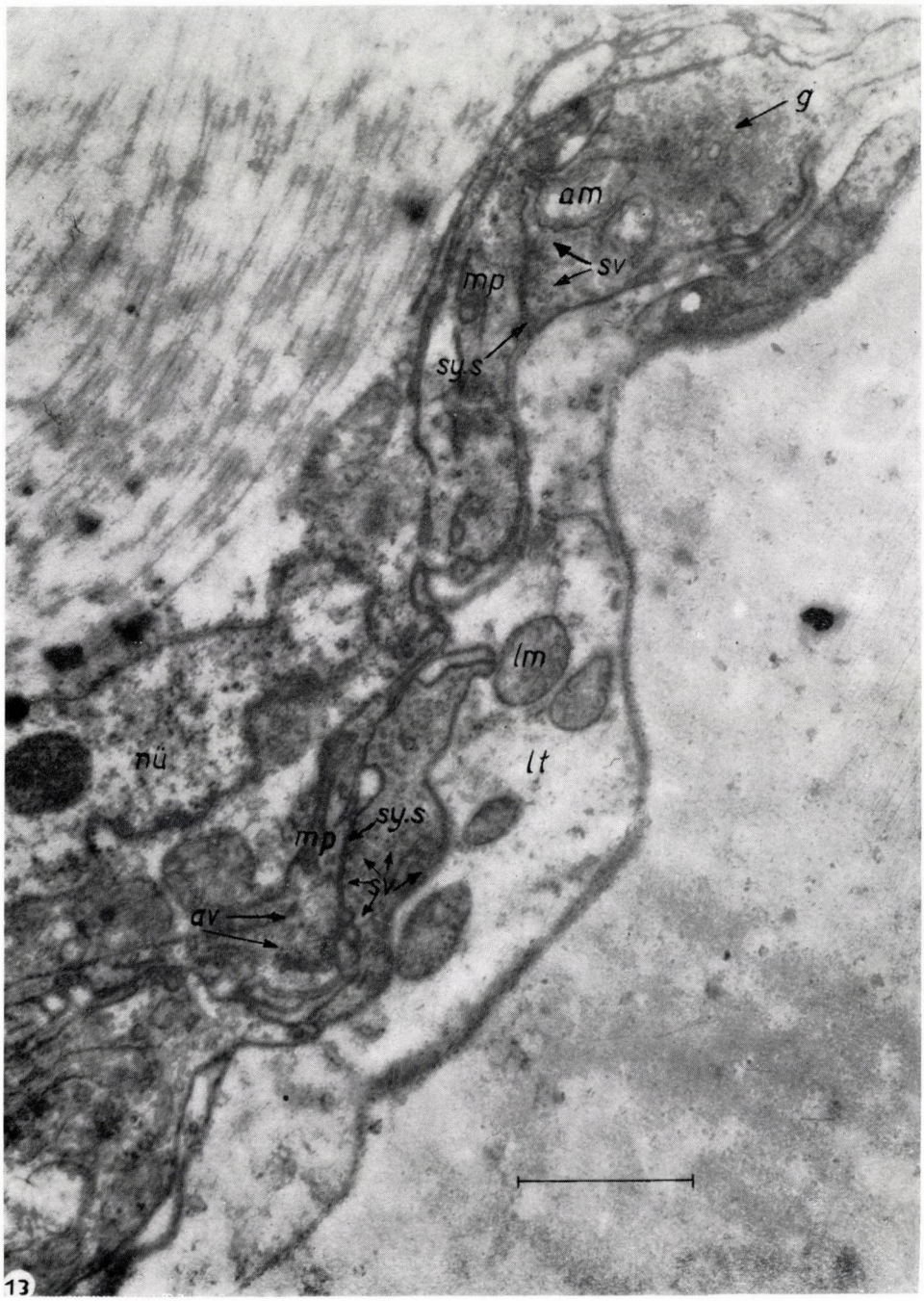


Fig. 13. Longitudinal section of end-plate with structural elements resembling a Golgi apparatus (*g*). *am* — axon mitochondria; *av* — aposynaptic vesicles; *lb* — lemmoblast; *lm* — lemmoblast mitochondria; *mp* — muscle pillars; *nu* — nucleus of the muscle fibre; —*sv* — synaptic vesicles; *sy. s* — synaptic surface. *Cetonia aurata*, × 23,000

Finally an observation should be noted that might throw some light on the origin of synaptic vesicles. Fig. 13 shows a longitudinal section of an end-plate in *Cetonia*. The whole terminal part of the axon is filled with the usual synaptic vesicles, in the centre of the lower thickened profile, however, there is a concentric membrane system closely resembling a Golgi apparatus. Unfortunately this preparation was not in the best state of conservation, nevertheless it conveys the impression that the synaptic vesicles might be derived from this membrane system.

We have not been able so far to ascertain the mode of termination, especially of contact established with the muscle of the small axons that are found in most limb muscle nerves.

Discussion

At this first step in a longer series of investigations it might probably be advisable to refrain from drawing far reaching functional conclusions. We want, therefore, only to point out certain characteristic features of the insect neuromuscular junction which, as will be seen later, in spite of considerable differences in structural details are common features of the architectural design.

(1) There is no such thing in insect muscle innervation as the "subneural apparatus" of the vertebrates; i.e. no folded region of the subsynaptic membrane. One could, of course, consider the muscular pillars as the analogues of the tops of the folds, where there is an immediate attachment to the axolemma — and the region between the pillars as the analogues of the furrows between individual folds. But besides the difference in the order of magnitude of these structures, a direct comparison of the two is difficult also from the histochemical viewpoint. The cholinesterases of the insect neuromuscular endings are neither specific (acetyl-)cholinesterase, nor butyrilcholinesterase of the vertebrates [6], but of a different kind. Moreover their localization is presynaptic and probably to some extent lemmoblastic rather than postsynaptic.

(2) It is a remarkable property of the insect neuromuscular synapse, that only a small a fraction of the axolemmal surface is in fact attached directly to the postsynaptic muscle fibre membrane. This leads to another prominent feature of this type of synapse, the cysterns of intercellular spaces that separate most parts of the surface membranes of the presynaptic and postsynaptic elements. These cysterns could be very useful in explaining currents or extracellular ionic fluxes that are supposed to occur during impulse transmission. It would be, however, dangerous to draw far reaching conclusions from these observations, because — as we shall see in forthcoming papers — these intercellular cysterns are far less prominent and even almost

completely absent in other forms. As seen from Fig. 13, they are rather insignificant already in the end-plates of *Cetonia*. Apart from the existence of intercellular cysterns the region of "active" (?) contact between the axolemma and postsynaptic membrane is in all types of insect neuromuscular synapses restricted to highly specialized regions of the postsynaptic muscle part. — This might be related to the "multiterminal" character of insect muscle innervation where there is no necessity for impulse propagation from the end-plate region — as it is the case in vertebrate neuromuscular ending — due to the many places along the muscle fibre that have immediate contact with nervous elements.

(3) The postsynaptic region of the muscle fibre is highly specialized in all types of insect muscle innervation. This has been correctly described already by EDWARDS and co-workers. [3]. Although the structure of postsynaptic regions is relatively simple, as compared with other types of insect muscle innervation, the postsynaptic pillars are densely packed with aposynaptic granules; but even more significant are membrane systems that appear to originate in the base of the pillars. As to be shown in subsequent papers, this reticulum of membrane systems seems to be connected with (i) perifibrillar membranes, (ii) Z-membranes and occasionally with (iii) muscular mitochondria. From the viewpoint of the transmission of the excitatory process from the postsynaptic membrane to the myofibrils, the connexions of these postsynaptic membrane systems mentioned under (i) and (ii) might be of particular interest.

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ELEKTRONENMIKROSKOPISCHE STUDIEN ÜBER DIE MUSKELINNERVATION DES ENDPLATTEN-TYPUS BEI INSEKTEN

Der submikroskopische Bau neuromuskulärer Endplatten der Faser-(Bein-)Muskulatur bei *Apis mellifica* (Hymenoptera), *Geotrupes vernalis* und *Cetonia aurata* (Coleoptera) wird beschrieben. Dieser Typus der Endplatten ist in der Faser-Muskulatur von *Hymenopteren* und *Coleopteren* recht allgemein und zugleich ein einfacherer Fall des sogen. »Endplattentypus«. Der tatsächliche Kontakt zwischen Axonendstreck- und Muskelfaser wird durch pfeilerförmige Auswüchse der Muskelfaser hergestellt, die von zwei Seiten alternierend an die längs der Muskelfaser parallel verlaufenden Axon-Endstücke von beiden Seiten herantreten und mit denselben mittels einer synaptischen Doppelmembran verbunden sind. Alle übrigen Teile der Oberfläche der Nervenendigung sind durch eine dünne Zellschicht der Nervenscheidenzellen dicht umschlossen und ausserdem von der Oberfläche der Muskelfaser noch durch ein kompliziertes Labyrinth extrazellulärer Zysternen getrennt. Einige bedeutsamere strukturelle Eigenheiten der zellulären Nervenscheidenelemente, der Nervenendigung und besonders des postsynaptischen Gebietes der Muskelfaser werden eingehender beschrieben und ihre mutmassliche funktionelle Bedeutung kurz besprochen.

ЭЛЕКТРОННОМИКРОСКОПИЧЕСКОЕ ИССЛЕДОВАНИЕ МИОНЕВРАЛЬНЫХ СИНАПСОВ У НАСЕКОМЫХ

На основании электронномикроскопических исследований автор описывает ультраструктуру иннервации мышц ножек *Apis mellifica* (Hymenoptera), *Geotrupes vernalis*, *Cetonia aurata* (Coleoptera). Иннервацию можно причислить к синаптическому типу моторных бляшек, к их самой простой форме.

В образовании действительной, относительно небольшой соприкасающейся (синаптической) поверхности участвуют поверхность аксона, обращенная к мышце, и «мостики» мышцы, выступающие попеременно с обеих сторон. Аксон, за исключением небольшой синаптической поверхности, покрыт везде леммобластом. Поверхность аксона, покрытая леммобластом, отделена от саркоплазматической мембраны на территории нервных бляшек очень сложной, так называемой системой интерцеллюларных цистерн. Пространственная реконструкция этого типа нервных бляшек изображена на рисунке 3.

Вконце автор пытается дать функциональное объяснение леммобласта, окончания аксона и постсинаптической области.

JÓZSEF HÁMORI, Budapest IX., Tüzoltó u. 58, Hungary.

RECENSIONES

D. R. PITELKA: **Electron-microscopic structure of Protozoa**

Pergamon Press, Oxford (1963). 269 pp., 84 s.

The rapid development of electron microscopy brought about an increased interest in the fine structure of all types of biological material. Protozoa which fascinated biologists using light microscope for many years were of course included in the list of objects studied under the new instrument. Their study revealed a great number of interesting new features of protoplasmic organization and at the same time supplied new support to the idea concerning basic similarities in the structural composition of different kinds of life at the cellular level. The intense research work resulted in a long series of publications scattered in various cytological, parasitological, zoological and medical journals that badly needed to be summarized and critically evaluated. The book under review is a welcome and highly successful attempt of this critical evaluation.

An introductory chapter is followed by a detailed treatment of the protozoa as cells with a comparison of different cell organelles as found in protozoa and metazoan cells. The findings reviewed in this chapter clearly reveal that the basic tasks of protoplasmic functions are realized in essentially similar ways in different organisms. This unity of cellular organization over both kingdoms of life is one of the most powerful arguments in favour of monophiletic origin of life. The greatest part of the book presents the detailed findings on individual species grouped according to the major protozoan taxons. These chapters remarkably show the great diversity of protozoan forms of life. Almost all major groups have been studied under the electron microscope as yet, but there is an enormous number of interesting and important species still awaiting study. A final chapter summarizes the main features of protozoan body and gives an outlook for future work.

88 well selected and executed electron micrographs illustrate the text. The comprehensive list of references extends over 27 pages.

The book is an indispensable basic starting point for all future workers in electron microscopy of protozoa and can be warmly recommended to all protozoologists and cytologists.

MIKLÓS MÜLLER (Budapest)

M. FINGERMAN: **The control of chromatophores**

Pergamon Press, Oxford (1963), IX, 184 pp., 50 s.

FINGERMAN, professor of zoology at Newcomb College, Tulane University (New Orleans), is known to have been engaged for many years in research work concerning changes in the pigmentation of crabs and the mechanism of such changes. Our author began his career at the side of F. A. BROWN JR., a likewise reputed scientist in this field.

FINGERMAN's book under rewiew is not the first work on the subject, nor can it be said that it is unique in its class; however, the work has no such pretentions since the principal object of the author was to present the most recent results and hypotheses regarding the subject indicated by the title.

The book utilizes data from 475 papers and consists of 7 chapters.

Chapter 1 covers 18 pages and deals with the types, chemistry and classification of chromatophores, the functional significance of colour changes. It presents a concise survey of the current notions and theories regarding chromatophores and colour changes.

Chapter 2, the longest of the book, covers 66 pages and is devoted to the chromatophores of crabs. The origin of chromatophores and their chemical composition are discussed in the first part of the chapter, while the rest thereof deals with the chromatophores and their control in the Isopoda, Brachyuria, Astacidae and Stomatopoda.

Chapters 3, 4 and 5 deal with the chromatophores of the Insecta (8 pp.), Cephalopoda (4 pp.) and Echinodermata (3 pp.). The very brevity of the chapters betrays the deficiency of our knowledge regarding these groups of animals.

Chapter 6 (43 pp.) deals with the chromatophores of vertebrates. It begins with a general discussion concerning the origin and chemistry of chromatophorotropins and goes on to treat of the chromatophores and their control in the Cyclostomata, Elasmobranchii, Teleostei, Amphibia and Reptilia.

Chapter 7, the last and shortest of the book ("Respectives"), summarizes the characteristic features of this valuable monograph.

The book offers an interesting and clear survey of results achieved during the last 15 years. Very aptly, the author occasionally goes beyond a simple listing of the often contradictory physiological data and tries to coordinate them. The work under review is a successful representative of tendencies toward bridging the wide gap between the two primary divisions of the animal kingdom.

The work would have gained in value if the author had — however briefly — included in it all animals which are known or supposed to be provided with chromatophores and the mechanism regulating them. Chapter 2 might thus have been extended to the Entomotraca and Malacostraca.

There can be no doubt that the monograph, with its illustrations and indexes, is a valuable work and might stimulate further fruitful research work.

István Konok (Tihany)

Printed in Hungary

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AMINO ACID METABOLISM AND RESISTANCE TO USTILAGO MAYDIS (DC.) CD. IN MAIZE

M. PETHŐ
COLLEGE OF AGRICULTURE, DEBRECEN

(Received July 20, 1963)

Synopsis

A paper chromatographic study of amino acid content of healthy and diseased maize plants infected with *Ustilago maydis* was undertaken at various stages of disease development. Maize varieties exhibiting various degrees of resistance to *U. maydis* were included in the experiments. A negative correlation between amino-acid content of healthy plants and their resistance to infection was established. In gall tissues of infected plants a marked accumulation of aspartic and glutamic acid was observed, particularly in the susceptible variety. The serine and glycine content markedly decreased in the susceptible variety. The alanine content decreased in all varieties tested; the greatest decrease characterized the less susceptible variety. The infection induced a rise in keto-acid level in gall tissues. The effect of infection was perceptible in the metabolism of neighbouring healthy leaves as well. On the basis of the data presented it is postulated that the infection results in the stimulation of biosynthetic mechanisms and this leads to an accumulation of keto acids and amino acids providing carbon skeletons for the intense protein synthesis of developing fungal hyphae and for proliferation of gall tissue. The differences in susceptibility might partly be explained by the different capacities of these biosynthetic systems to operate upon parasitic attack. In addition, the preinfectional amino acid level of the young tissues appears to play a role in resistance.

Introduction

It was found in previous studies [12] that the dicaryotic stage of *Ustilago maydis* is able to grow only in the presence of certain amino acids. Of the examined amino acids, glycine, alanine and serine proved to be the most effective. In the presence of inorganic N-sources the amino acid requirement of the dicaryotic hyphae of *U. maydis* is not highly specific. The growth of hyphae in synthetic media is ensured by a number of amino acids.

As the parasitic stage of the fungus is unable to synthesize amino acids from inorganic N-compounds, an important manifestation of parasitism in *U. maydis* is the utilization of amino acids present in the host tissues. It was found [13] that the decrease of amino acids in the gall was due to that they were necessary for the development of the hypertrophic tissue and were, on the other hand, utilized by the fungus for its proliferation at the site of infection.

In the leaf tissues of the young maize plant alanine is the predominant amino acid [14]. It contains further considerable quantities of serine, glycine and glutamic acid. The amount of other amino acids is insignificant. It is, therefore, natural that it is chiefly the amount of alanine, serine and glicine

which decreases in the infected tissues. The level of glutamic acid in the gall tissue increased in all examined varieties. Concerning glutamine the picture is more confusing. DeVay and Rowell [4] reported on a lowered level of glutamine in Ustilago-induced galls of young maize plants. By contrast, Turian [20] observed increased glutamine, glutamate and keto-acid levels in galls collected in the field.

It was after such antecedents that the present detailed investigations were started concerning the amino acid metabolism of maize plants infected with *U. maydis*. These investigations had the following aims.

(1) To study the effect of the infection on the level of amino acids in diseased tissues, in the healthy tissues surrounding the galls, and in tissues at a greater distance from the site of infection.

(2) To carry out these investigations at different stages of the disease.

(3) To ascertain the possible correlation between amino-acid content and susceptibility. To this end the amino-acid content of various maize varieties exhibiting different degrees of resistance to infection with *U. maydis* was studied.

(4) To study the effect of infection on differently susceptible varieties.

Material and methods

The experiments were carried out with the maize variety "Mindszentpusztai sárga lófogú", moderately susceptible to *U. maydis* ; with the inbred line "42-313", hardly susceptible to infection; and with the extremely susceptible inbred line "62-411". The inbred lines were produced from local varieties. Sporidia of compatible strains of *U. maydis*, obtained from a 36-hour culture, were subcutaneously injected near the growing point. The first samples were collected 14 days after infection, just after the appearance of young galls. Samples were taken also at later stages in order to study the relationship between hosts and parasites in different phases of the disease.

The tissues were extracted with alcohol (80 per cent final concentration). The content of total free amino acids was determined by the procedure of Kisfaludy and Braun [10] modified as follows: 0.15—0.30 ml of the alcoholic extract was added to 4 ml freshly prepared ninhydrin reagent (60 mg $SnCl_2 \cdot 2H_2O$ + 50 ml acetate buffer pH 4.7 + 0.75 g ninhydrin + 50 ml ethyleneglycol); the tubes were shaken, then left standing for a few minutes, after which we placed them in a boiling waterbath for 20 minutes. This was followed by cooling in ice-water for 3 minutes. The reaction mixture was filled up to 10 ml with a 7 : 3 mixture of acetone and water. After 15 minutes, the amino-acid content was determined by colorimetry. A calibration curve prepared from alanine served as standard.

The concentration of various amino acids was determined by paper chromatography after desalting the extracts on a Varion K.S. cation exchange column and elution by 2 *N* NH_4OH. The eluates were evaporated to dryness on a waterbath and the residue was taken up in 30 per cent isopropanol. Descending paper chromatography was applied (filter paper: Whatman No. 4), and the mixture was run in a system composed of phenol and water (100 : 20). The chromatograms were developed with 0.1 per cent chloroform-containing ninhydrin solution. The quantitative determination of amino acids was carried out by visual comparison of spot size and colour intensity with a standard series of amino-acid concentrations [8].

Keto acids were determined according to Friedemann and Haugen [7].

The results are presented on a fresh-weight basis. Average values obtained from the analysis of at least 3 plant individuals are given.

Results

Free amino acids in maize varieties exhibiting different degrees of susceptibility to U. maydis

The total free amino-acid content of the three maize varieties included in the experiments is shown in Fig. 1. It can be seen that the amino-acid content is highest in the leaf blades of variety No. "42" which is the most resistant to infection with *U. maydis*. The lowest amino-acid level was found in the most susceptible variety ("Mindszentpusztai sárga"). It seems important

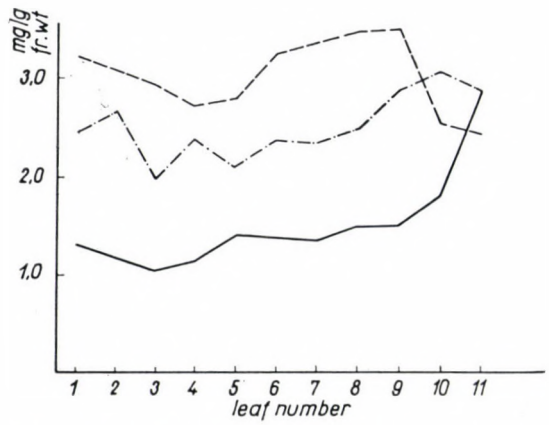

Fig. 1. Free amino-acid content in healthy leaves of three maize varieties of different susceptibilities to *Ustilago maydis* (———) local variety No. 62; — — — local variety No. 42; —.—.— "Mindszentpusztai sárga"). The leaves are numbered acropetally

that in very young leaves the relation between amino-acid content and resistance is just the opposite. At this stage the leaves of the most susceptible variety show the highest amino-acid levels. Therefore, the investigations were extended, and the amino-acid content of very young leaves of six maize varieties was determined. In Fig. 2 the correlation between resistance and amino-acid content can be seen. Higher degree of susceptibility is associated with a higher amino-acid level.

In Fig. 3 the levels of five amino acids in the leaves of the three varieties investigated in detail are given. The amino-acid contents of the lower (I), middle (II) and upper (III) leaves are given separately. The leaves of group II correspond to the leaves bearing galls in the infected plants.

It can be seen from Fig. 3 that alanine is the major amino acid. The most susceptible variety (No. "62") contains the smallest amounts of amino acids, including alanine.

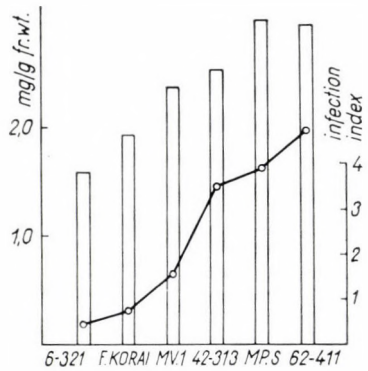

Fig. 2. Free amino-acid content (columns) in the young leaves of six maize varieties, and its relation to susceptibility (continuous line). Susceptibility is given as "the infection index" = number of diseased plants multiplied with the degree of infection and divided by the number of infected plants. 6—321 = "Red King Felsőszentiváni"; 42—313 = "Iregszemcsei"; 62—411 = "Újszentiváni" 3-year old inbred lines of local varieties. MV. 1. = "Martonvásári 1. hybrid"; MP.S. = "Mindszentpusztai sárga"

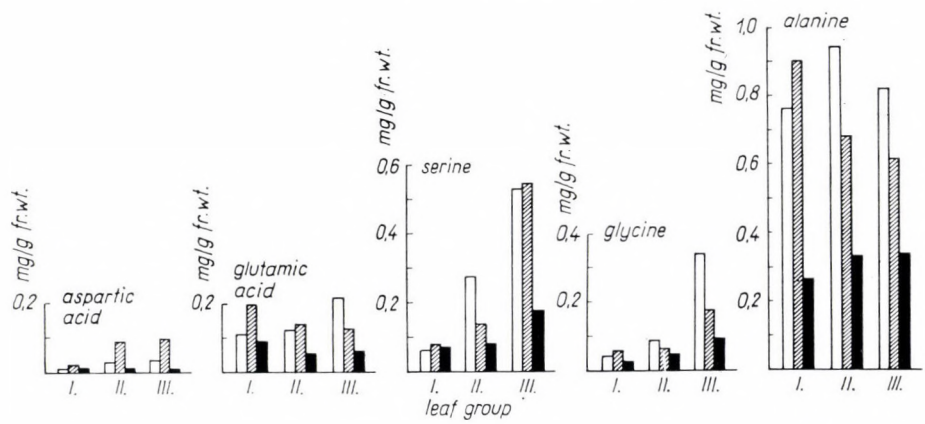

Fig. 3. Free amino-acid content in the leaf blades of three maize varieties of different susceptibilities to *Ustilago maydis*. I. = lower, II. = middle, III. = upper leaves (cf. text). Empty column = local variety No. 42. Striated column = "Mindszentpusztai sárga". Black column = local variety No. 62

Effect of infection on the amino-acid content of the highly susceptible variety "No. 62" at different stages of the disease

It can be seen in Fig. 4 that, after an initial increase, the amino-acid content of the galls decreased and that the amino acid supply of the host tissues was practically exhausted 28 days after infection. This may have been due to the utilization of amino acids by the fungus.

Only minor changes were observed in the amino-acid content of the "healthy" parts of gall-bearing leaves. Simultaneously, amino acids accumu-

lated in the lower leaves. In leaves of higher insertions (above those bearing galls), after some initial decrease, the amino-acid level also increased.

The qualitative analysis of amino-acid content, in line with previous results [14], indicated that the various amino acids were affected differently by the infection. This is shown in Table 1.

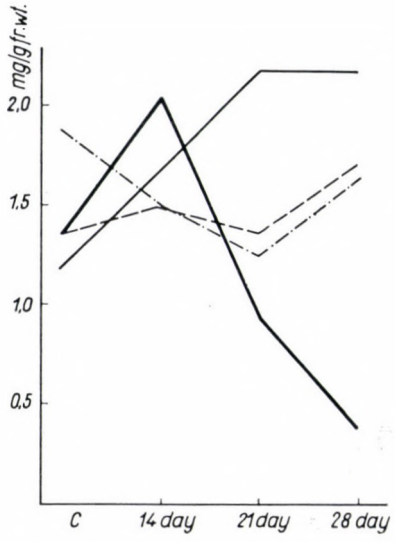

Fig. 4. Effect of infection with *Ustilago maydis* on the free amino-acid content of the local variety No. 62, 14, 21 and 28 days after infection. (——— gall tissue; — — — uninfected part of gall-bearing leaves; ——— leaves below the infected ones, —.—.— leaves above the infected ones

In the leaves below the infected ones, the amino-acid level (except the concentration of alanine) was not altered to any marked extent. The alanine level increased upon infection.

In leaves above the infected ones, a highly increased serine level was found 14 days after infection. In contrast, the glutamic-acid and alanine concentration decreased. Later, also the serine level decreased. High serine levels are characteristic for young leaves in general. Therefore, the high serine content of the leaves in question might be explained with the reduced growth of infected plants [15]. Due to the reduced growth of infected plants, the leaves above the galls are "younger" than the corresponding leaves of healthy plants.

The effect of infection on the amino-acid level is strongest in the infected leaves. An increase in aspartic-acid concentration is characteristic both for the galls and for the tissues of gall-bearing leaves. As can be seen in Fig. 5, in young galls the concentration of glutamic acid was also highly increased (700%) as compared to the glutamic-acid level in comparable healthy leaves.

Table 1

Effect of infection with Ustilago maydis *on the amino-acid content of the leaves of the highly susceptible maize variety No.* 62*

(a) 14 days after infection

Amino acid	Lower leaves (1st group)			Middle leaves (2nd group)						Upper leaves (3rd group)		
	K	eg1		K	f		g			K	eg2	
	mg/g	mg/g	eg/K	mg/g	mg/g	f/K	mg/g	g/K	g/f	mg/g	mg/g	eg/K
Aspartic acid .	+	0.025	+	+	0.106	++	0.286	++	2.69	+	0.038	+
Glutamic acid .	0.091	0.084	−1.08	0.050	0.243	4.86	0.353	7.06	1.45	0.056	0.027	−2.07
Serine	0.041	0.041	1.00	0.076	0.159	2.09	0.231	3.03	1.45	0.172	0.437	2.54
Glycine	0.023	0.030	1.30	0.044	0.043	−1.02	0.065	1.47	1.51	0.081	0.079	−1.02
Alanine	0.318	0.460	1.44	0.330	0.295	−1.11	0.223	− 1.47	− 1.32	0.336	0.322	−1.04
(b) 21 days after infection												
Aspartic acid .	+	0.012	+	+	0.054	+	0.061	+	1.12	+	0.009	±
Glutamic acid .	0.091	0.051	−1.78	0.050	0.152	3.04	0.196	3.92	1.28	0.056	0.012	−4.66
Serine	0.041	0.058	1.41	0.076	0.137	1.80	0.114	1.50	− 1.20	0.172	0.152	−1.13
Glycine	0.023	0.025	1.08	0.044	0.058	1.31	0.023	− 1.91	− 2.52	0.081	0.073	−1.10
Alanine	0.318	0.721	2.26	0.330	0.309	−1.06	0.161	− 2.04	− 1.91	0.336	0.180	−1.86
(c) 28 days after infection												
Aspartic acid .	+	+	±	+	0.016	+	+	±	−	+	0.011	+
Glutamic acid .	0.091	0.065	−1.40	0.050	0.035	−1.42	0.111	2.22	3.17	0.056	0.036	−1.55
Serine	0.041	0.051	1.24	0.076	0.084	1.10	0.033	− 2.30	− 2.54	0.172	0.157	−1.09
Glycine	0.023	0.044	1.91	0.044	0.092	2.09	0.010	− 4.40	− 9.20	0.081	0.085	1.04
Alanine	0.318	1.100	3.45	0.330	0.634	1.92	0.030	−11.00	−21.13	0.336	0.327	−1.02

* K = leaves of control plants; eg1 = leaves under the infected ones; eg2 = leaves above the infected ones; f = "healthy" tissue adjacent to the gall; g = gall; eg/K, f/K and g/K = relative amino acid contents.

In the later stages of the disease, a decrease in the amount of aspartic acid and glutamic acid also contributed to a general decrease in the level of amino acids in the galls (Fig. 4). However, decrease was most conspicuous in respect of the other three examined amino acids.

The level of serine and glycine was initially high in both the galls and in the tissues of gall-bearing leaves, but decreased in the later phases of the

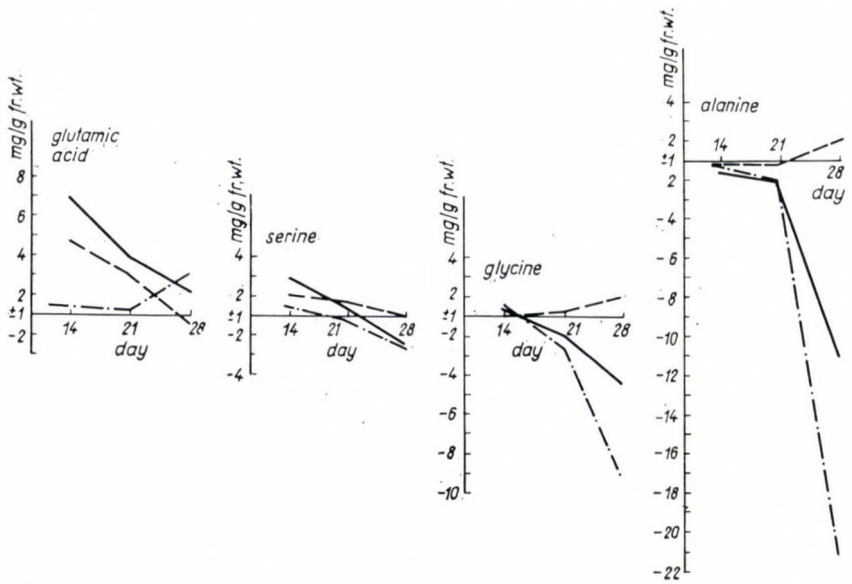

Fig. 5. Amino-acid content of infected plants of variety No. 62 in various phases of the disease (14, 21 and 28 days after infection). Relative amino-acid content of the galls (———) and gall-bearing leaves (— — —) as compared to corresponding control leaves (horizontal line), and relative amino-acid content of the galls (—. —. —), as compared to the gall-bearing leaves (horizontal line)

disease. By contrast, the concentration of alanine was low even in the gall tissues throughout the infection.

The observed picture might be explained by two processes taking place simultaneously in infected tissues:

(*a*) Increase in amino acid synthesis upon infection.

(*b*) Synthesis of proteins of the gall tissue and of the fungal mycelia from the free amino acid pool.

These tow processes lead finally to a decrease of free amino acids in the leaves of the maize.

It was shown in a previous study [13] that the level of reducing sugars is increased in the gall tissue. This might be related to the higher respiratory rate of the gall. The increased rate of amino-acid synthesis is probably correlated with the stimulated respiration which provides the carbon sceletons (keto acids) for amino-acid synthesis. The increased glutamic-acid level of the galls

is in line with this idea. The other amino acids probably arise secondarily by transamination. The low alanine content remains unexplained unless the synthesis of alanine is primarily a photosynthetic process. The gall tissue contains little chlorophyll.

To sum up, it appears that the fungus induces a stimulated metabolism in the host tissues and uses the products of stimulated metabolism for the synthetic processes necessary for fungal growth. The parasitically induced alterations are not localized to the immediate neighbourhood of the infection site.

The effect of infection on the amino-acid content of maize varieties exhibiting various degrees of resistance to Ustilago maydis

Results obtained with three varieties are summarized in Fig. 6. It can be seen that the concentration of amino acid decreased in the leaf tissues of the more or less resistant and of the moderately susceptible variety, both in the

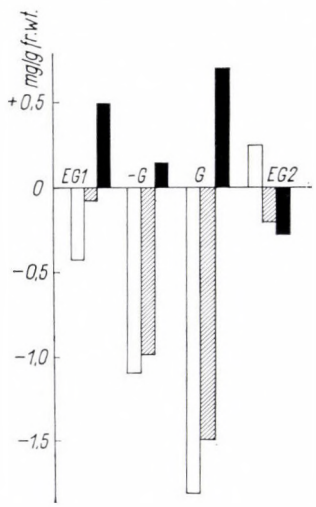

Fig. 6. Differences in the amino-acid content of similarly situated infected and healthy leaves. EG1 = leaves below the infected ones. EG2 = leaves above the infected ones. -G = "healthy" parts of gall-bearing leaves. G = gall tissue. Empty column = local variety No. 42. Striated column = "Mindszentpusztai sárga". Black column = local variety No. 62

uninfected part of the gall-bearing leaves and in leaves below infected ones. Determinations were made 14 days after infection. The amino-acid content increased in the corresponding tissues of the highly susceptible variety. The trend of the change in the amino-acid level was the same in the gall tissue.

Post-infectional changes in the level of various amino acids are shown in Tables 1 and 2 and Fig. 7. In the figure, the change in concentration (increase

Table 2

Effect of infection with Ustilago maydis *on the amino-acid content of moderately susceptible maize varieties**

Local variety No. 42

Amino acid	Lower leaves (1st group)			Middle leaves (2nd group)						Upper leaves (3rd group)		
	K	eg1		K	f		g			K	eg2	
	mg/g	mg/g	eg/k	mg/g	mg/g	f/K	mg/g	g/K	g/f	mg/g	mg/g	eg/K
Aspartic acid .	+	+	±	0.029	0.188	6.48	0.170	5.86	—1.10	0.032	+	— —
Glutamic acid .	0.117	0.138	1.16	0.127	0.245	1.92	0.216	1.70	—1.13	0.213	+	— — —
Serine	0.059	0.012	—4.91	0.268	0.111	—2.41	0.144	—1.86	1.29	0.526	0.870	1.65
Glycine	0.040	0.012	—3.30	0.083	0.088	1.06	0.101	1.21	1.14	0.323	0.261	—1.23
Alanine	0.761	0.831	1.09	0.938	0.654	—1.43	0.187	—5.01	—3.49	0.814	1.044	1.28
„Mindszentpusztai sárga"												
Aspartic acid .	0.017	0.026	1.52	0.089	0.294	3.30	0.125	1.40	—2.35	0.097	0.020	—4.85
Glutamic acid .	0.200	0.148	—1.35	0.143	0.307	2.14	0.190	1.32	—1.61	0.119	0.068	—1.75
Serine	0.074	0.056	—1.32	0.137	0.345	2.51	0.062	—2.20	—5.56	0.542	0.500	—1.08
Glycine	0.057	0.040	—1.42	0.062	0.124	2.00	0.032	—1.93	—3.87	0.177	0.185	1.04
Alanine	0.901	0.795	—1.13	0.681	0.447	—1.52	0.090	—7.56	—4.96	0.608	0.721	1.18

* For legends see Table 1.

or decrease) is given with reference to corresponding healthy leaves. It can be seen that in all the three varieties the level of aspartic and glutamic acid increased both in the non-infected part of the gall-bearing leaves and in the galls. The greatest increase was found in highly susceptible variety No. 62.

The serine content was reduced in the galls and in the uninfected parts of gall-bearing leaves in variety No. 42. In the variety "Mindszentpusztai sárga", the serine content decreased in the gall tissues only. In the highly susceptible variety there was a general increase in serine content.

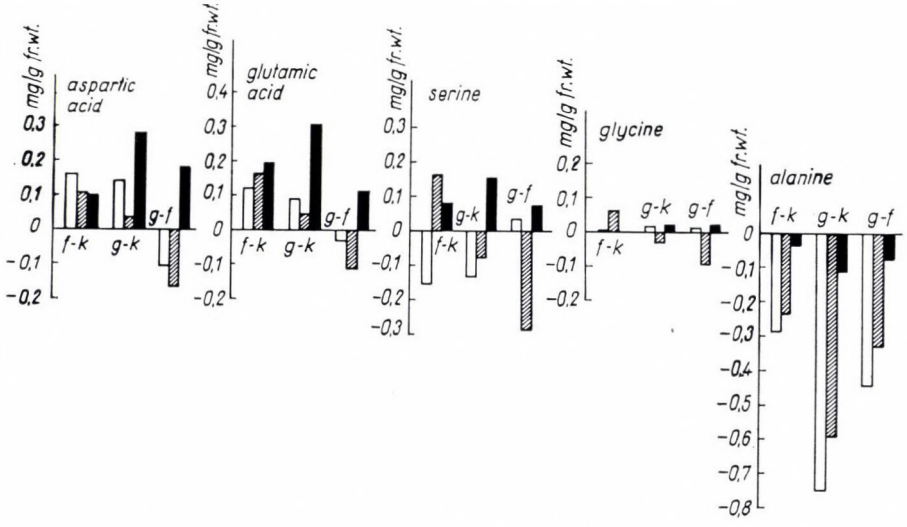

Fig. 7. Effect of infection on the amino-acid content of maize varieties. The columns represent plus and minus differences between the amino-acid content of "healthy" parts of gall-bearing leaves and control leaves (f—k), gall tissues and control leaves (g—k), gall tissues and "healthy" parts of gall-bearing leaves (g—f). Empty column = local variety No. 42. Striated column = "Mindszentpusztai sárga". Black column = local variety No. 62

The glicine content underwent no marked change. By contrast, the alanine level decreased in the infected leaves of all the three varieties. The greatest decrease in alanine was found in the galls. This may have been due to a process of rapid utilization. The decrease in alanine was smallest in the highly susceptible variety in all examined tissues. The increased utilization of alanine was there probably somewhat masked by increased alanine synthesis.

The differences in amino-acid content in infected and healthy tissues, and the varietal differences are shown in the column g/K of Table 1*a* and Table 2. There was a marked post-infectional accumulation of glutamic acid in the susceptible variety. It appears that, in the susceptible variety, it is principally the synthesis of primary amino acid which is geared to a higher level.

In preliminary investigations, the effect of infection on the keto-acid level was also tested. The results are summarized in Table 3. It is evident that

the keto-acid content increased upon infection, particularly in the highly susceptible variety. Paper chromatographic studies indicated that the major component was α-ketoglutaric acid. This observation is in line with the theory that the intensity of primary amino-acid synthesis is increased upon infection.

Table 3

Effect of infection on the keto-acid content of maize varieties

Local varieties	Total keto-acid content mg/100 g tissue		c/g
	control (c)	gall (g)	
No. 62	2.12	6.15	2.90
No. 42	2.66	3.62	1.36

Infections localized to the stem also affect the amino-acid metabolism of the leaf tissues. As shown in Fig. 8, the amino-acid content in the leaf tissues decreased particularly in leaves above the gall developing on the internode. All amino acids were affected to approximately the same extent (Fig. 9).

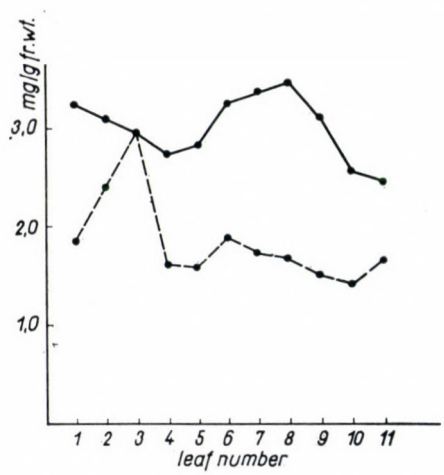

Fig. 8. Effect of galls developed on the 3rd and 4th internodes on the level of free amino acids in the leaves of the local variety No. 42. (——— control; — — — infected plant)

Discussion

As the parasitic phase of *Ustilago maydis* is unable to meet its amino-acid requirement by synthesis from inorganic N-sources [12], it seems justified to assume that the amino-acid metabolism of the maize plant plays a role in the resistance of the host to parasitic attack. However, no correlation was found

to exist between the amino-acid content of healthy plants and their resistance to *U. maydis*. This indicates that the relation between amino-acid level and susceptibility is complex. Older leaves are completely resistant to infection despite their relatively high amino-acid content. In younger leaves of susceptible varieties, however, the amino-acid content is high (Fig. 2), and the high amino-acid level of these leaves might have a role in their susceptibility. This possibility is particularly suggestive, as the major free amino acids in the maize leaf are identical with those shown to be effective in promoting the development of the parasitic phase of the fungus on synthetic media.

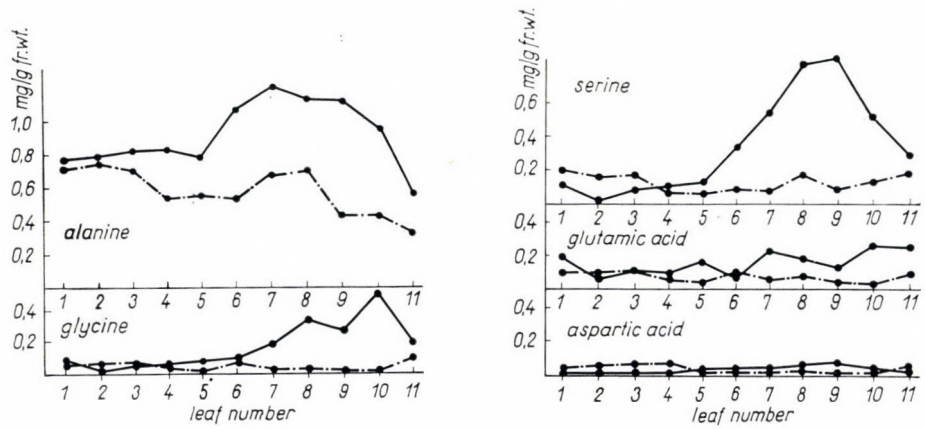

Fig. 9. Effect of galls developed on the 3rd and 4th internodes on the level of some amino acids in the leaf tissues of local variety No. 42. (——— control; —.—.— infected plant)

It has been shown by a number of authors that the nitrogen nutrition of the host has an important effect on the outcome of the host-parasite relationship [1, 3, 5, 14, 16, 18, 20, 21]. ALTEN and ORTH [1] demonstrated that an increased N : K ratio increased the concentration of free amino acids in the potato tissues and also their susceptibility to *Phytophthora*. Of course, not only the total amino-acid concentration is important but the change in amino-acid spectrum as well. Some amino acids might be inhibitory for the fungus even at low concentrations. This has been shown in previous studies on the *in vitro* growth of the fungus [12]. In this connection the selective inhibitory effect exerted by extracts from older leaves on the growth of the parasitic phase of fungus should also be mentioned.

The complexity of the situation is indicated by the fact that various diseases are characterized by a different relationship between nitrogen metabolism and resistance. Thus, there appears to be a positive correlation between amino-acid content and susceptibility to "brusone" in rice [21]. By contrast, cabbage varieties resistant to *Botrytis cinerea* contain 5—6 times higher levels of some amino acids than the susceptible varieties [16]. In addition, some amino

acids have no effect on the parasite *in vitro* but increase the resistance of the host if supplied to the tissues preinfectionally *in vivo* [11].

The rise in the level of aspartic and glutamic acids in young galls, as reported in the present paper, is in line with the earlier findings of TURIAN [20]. The increased level of α-ketoglutaric acid found by him and confirmed in the present study (Table 3) is a further important feature of the parasitically altered metabolism and indicates that a whole biosynthetic sequence is stimulated upon infection. It seems possible that the varietal differences in susceptibility described in the present paper can be explained with a different response to infection of this biosynthetic system.

In addition to the role of primary amino-acid synthesis, transaminations also govern the amino-acid level. Thus, it was shown by SHAW and COLOTELO [18] that the concentration of a great number of amino acids became higher in rust-infected susceptible wheat leaves. The higher level of glutamine may be due to the enhanced activity of glutamine synthetase and to a higher ammonia level [5]. It must be stressed that not only dicarboxylic amino acids may be involved in the transamination process. The mutual transformation of serine and glycine has also been demonstrated in maize [9].

In our experiments, a decrease in alanine concentration was found in gall tissues induced by *U. maydis*. This is in contrast with TURIAN's observations [20] who found an increase in the alanine level of young galls. It should be noted that the data available on amino-acid metabolism in the gall tissues of maize infected with *U. maydis* are contradictory. DEVAY and ROWELL [4] found no major alteration of the amino-acid spectrum except a decrease in glutamine. By contrast, TURIAN [20] reported on a marked increase in glutamine in Ustilago-induced galls. These discrepancies might be explained by the fact that the various authors used plants of different susceptibilities in their studies, and the disease was differently advanced in their material.

It is important to stress that the effect of infection extends to the uninfected parts of the plant. The obviously toxic effect of the fungus has been observed by other authors also [17].

Infection of the stem also induces a decrease of amino acids in the leaves (Fig. 8). The fact that the concentration of all examined amino acids diminished to the same extent (Fig. 9) suggests that probably some early steps of amino-acid synthesis were affected. It is possible that the galls utilize the inorganic N-resources of the plant for the synthesis of amino acids. Therefore, the physiological effect of infection manifests itself in a different way in the infected leaves and in those above or below the galls.

In addition to the synthesis of keto acids [20], a sufficiently high inorganic N-level is necessary for increased amino-acid synthesis at the site of infection. As 80 to 95 per cent of the N-compounds taken up by the roots are transformed already in the root system in organic form [2], it seems possible

that the inorganic nitrogen content of the overground organs hampers intensive amino-acid synthesis in the infected tissues. Differences in the levels of free amino acids in the investigated maize varieties may be in connection with differences in the metabolic activity of their root systems. It is well known that, in the maize, there is a close correlation between the growth of the root system and that of overground organs [6].

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AMINOSÄURESTOFFWECHSEL UND RESISTENZ GEGEN USTILAGO MAYDIS BEI DER MAISPFLANZE

Verfasser untersuchte papierchromatographisch den Aminosäuregehalt von verschiedenen gesunden und infizierten Individuen von *Zea mays*, die gegen *Ustilago maydis* einen unterschiedlichen Resistenzgrad aufwiesen. Es wurde festgestellt, dass zwischen dem Aminosäuregehalt und der Resistenz gegen *Ustilago maydis* der gesunden Individuen der verschiedenen Sorten ein negativer Zusammenhang besteht. In den Gallen der infizierten Pflanzen steigt der Asparagin- und Glutaminsäuregehalt an. Dieser Prozess ist bei der untersuchten anfälligen Sorte mehr ausgeprägt. Der Serin- und Glycingehalt nimmt nur bei der anfälligen Sorte ab. Der Alaningehalt sinkt bei den untersuchten Sorten, besonders aber in den jungen Gallen der weniger anfälligen Sorten; bei der stark anfälligen Sorte tritt die Abnahme erst später ein. Unter dem Einfluss der Infektion steigt auch der Ketosäuregehalt in den Gallen an. Die physiologische Wirkung des Pilzes erstreckt sich auf die Gallen tragenden Blatteile und auch auf die gesunden Blätter. Verfasser nimmt auf Grund der Versuchsangaben an, dass es sich in den infizierten Geweben um eine allgemeine Stimulation eines biosynthetisierenden Systems handelt, die an Hand des erhöhten Ketosäure- bzw. Aminosäuregehaltes die intensive Eiweisssynthese, die zur stärkeren Proliferation der infizierten Gewebe und zum Pilzwachstum nötig ist, ermöglicht. Die unterschiedliche Anfälligkeit der Sorten kann durch die abweichende Aktivität dieses biosynthetisierenden Systems erklärt werden. Die unterschiedliche Resistenz wird aber auch durch den Gehalt an freien Aminosären von jungen infizierbaren Geweben beeinflusst.

АМИНОКИСЛОТНЫЙ ОБМЕН И УСТОЙЧИВОСТЬ КУКУРУЗЫ ПРОТИВ USTILAGO MAYDIS

При помощи метода хроматографии на бумаге автор изучал аминокислотный состав здоровых и пораженных особей сортов кукурузы, в различной степени восприимчивых к Ustilago maydis, на различных этапах взаимосвязи хозяина и паразита. Установил, что между аминокислотным составом здоровых особей отдельных сортов и их восприимчивостью к головне имеется отрицательная связь. В желваках пораженных растений повышается содержание аспарагиновой и глютаминовой кислот. У сильно восприимчивых сортов это повышение более значительное. Содержание серина и глицина снижается только у сильно восприимчивых сортов. В испытанных сортах наблюдается снижение содержания аланина, особенно в желваках менее восприимчивых сортов, а у сильно восприимчивых это снижение происходит только позже. Под влиянием заражения в желваках повышается также и содержание кетокислот. Физиологическое действие грибка распространяется и на части листьев, пораженных головней, а также и на незараженные листья. На основании полученных данных автор предполагает, что в зараженных тканях имеет место общее стимулирование определенной биосинтетизирующей системы, что в свою очередь путем повышенного содержания кето- и аминокислот обеспечивает условия для интенсивного синтеза белка, необходимого для интенсивной пролиферации зараженной ткани и для размножения гиф грибка. Наблюдаемая между восприимчивостью отдельных сортов разница объясняется неодинаковой активностью этой биосинтетизирующей системы, но повидимому находится также и под влиянием содержания свободных аминокислот молодых восприимчивых тканей отдельных сортов.

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WATER-SOLUBLE MELANIN PREPARATION

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Synopsis

For studies concerning the biology of melanotic tumours, melanin soluble in neutral watery medium was needed. Solubility is mainly ensured by the protein component linked to the pigment. A "neutral" melanin preparation from the pigmented layer of the bovine eye was obtained with the method of HOHENADL and DE PAOLA by washing in distilled water, but it was not soluble. It has been possible to isolate, by means of vacuum exsiccation and dialysis, a substance from the urine of melanomatous patients which was reversibly soluble in distilled water. The total nitrogen content of this melanin preparations was 6.6 per cent. By means of the ninhydrin reaction, 11 amino acids were demonstrated in its hydrochloric-acid hydrolysate by paper chromatography, three other spots were detected by treatment with ammoniated silver solution, and an unidentified yellow spot was detected by the ferri-ferri-cyanide reagent. The light-absorption curve of the urinary melanin is in many respects similar to that of artificial melanin prepared from tyrosine.

Introduction

Investigations into the biology of malignant melanotic tumours require studies to be made of the properties of melanin pigment. A serious difficulty in such studies is the fact that both the melanin injected subcutaneously and the melanin depot created by intramuscular injection remain unabsorbed and exert only a local effect.

According to HARANGHY and co-workers [6] melanin would nevertheless condition the effect of cancerogenic substances even in subcancerogenic doses. The problem could be best approached by the use of a soluble form of melanin, especially by injection through the intravenous route, and therefore the main task was to develop a suitable melanin preparation and to dissolve it in a medium not noxious for the organism. HARANGHY [5] recommends 10 per cent potassium carbonate as solvent. It is, according to PEARSE [8], absolutely insoluble in organic solvents or in any other solvent harmless for the tissues.

GJESDAL [3] has described a few "gentle" procedures by means of which "native" melanin could be prepared. The essence of these methods is to mechanically destroy the cells containing melanin, extract them with solutions of biological pH, then to salt out the pigmented fractions with ammonium sulphate. Melanin in such preparations is linked to a protein of pseudoglobular

nature, and this protein ensures the stability of the suspensions which can be prepared with water or with buffers of about pH 7. The melanin of hair and feathers is a keratinous inclusion which requires intense alkaline hydrolysis. Media with pH far from neutral denature or even hydrolyze the protein component of melanin. The solubility of pigments so prepared is considerably inferior to that of native preparations. When boiled with acid or base long enough, the protein component of melanin can be broken down completely, and the so-called rest pigment, which is in fact the compound we call melanin, is insoluble even in 1 n NaOH. In physical and chemical properties it becomes more and more similar to the humin substances and to mineral coals as a result of an increase in C and ash, and a decrease in H and N content.

Preparation of native melanin

To prepare native melanin, suitable for injection and stable also when suspended in water, we excised the pigmented layer of the bovine eye and suspended it in distilled water, according to HOHENADL and DE PAOLA [7]. The pigment fraction settles on centrifugation, the proteins dissolved in water and the floating residues of the membrane can be decanted. Resuspending the sediment and repeating purification 8 to 10 times, we get the ocular melanin preparation called "neutral", which, in the process of its preparation, has been exposed to no reagent other than distilled water. One third of this is boiled with azeotropic acid, an other third with 10 per cent KOH for 24 hours, under reflux cooling. On neutralization, melanin precipitates from the alkaline solution, and is not soluble in HCl. After centrifugation and washing several times in distilled water as well as drying in an exsiccator we obtain the bovine-eye melanin preparations subjected to "acid" or "alkaline" after-treatment.

In spite of the fact that it had been exposed to no kind of denaturing agent, the neutral bovine-eye melanin was not soluble in neutral aqueous medium. The alkaline preparation was also insoluble, and the acid preparation was downright hydrophobic. We tried, therefore, to use sunflower-seed oil as solvent and to disaggregate the acid preparation by exposure to ultrasound (0.2 W/square cm, for five minutes). A suspension stable for a few days was obtained, but when it was injected subcutaneously, foreign-body reaction developed around the pigment granules, proving that the material in the heterogenic phase was insoluble.

Preparation and properties of soluble melanin

Next, we collected urine from patients with melanoma as the material from which melanin suitable for injection could be prepared. In order to avoid effects leading to denaturation, we ought to have employed lyophilisation for

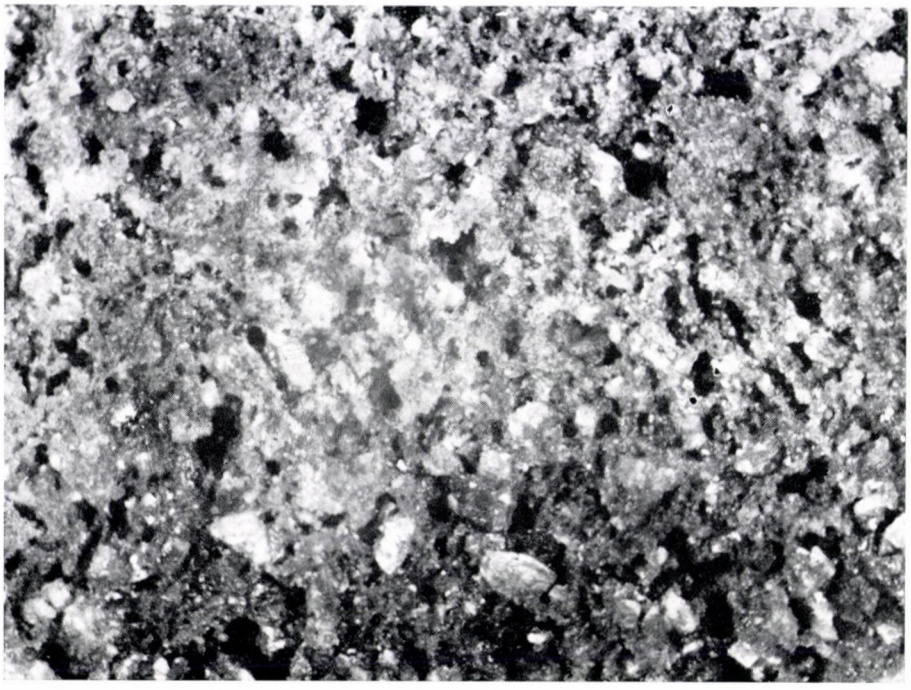

Fig. 1. Urine, from a melanuric patient, desiccated in a vacuum exsiccator (approx. natural size)

dehydration. Possessing no suitable equipment, we tried exsiccation in vacuo which saves the preparation from the effects of both heat and oxidation by air. However, the material kept foaming over, and so vacuum evaporation in CO_2 atmosphere proved unfeasible. Wanting to avoid the use of antifoam agents, we place the urine in H_2SO_4 vacuum exsiccator and dried it for 3 to 4 months, replacing the dehydrating agent several times. During that period, most of the proteins became putrid; urates and phosphates precipitated from the reddish brown sediment containing melanin, in the form of big, white crystals (Fig. 1).

The material thus obtained was suspended in water, poured into a dialyzing sheath and dialyzed against running tap water for 3 days. The dark brown solution was separated from the insoluble part by centrifugation and subjected to vacuum drying as described above. This second phase of dehydration lasted about 3 to 4 weeks. The residual substance coated the wall of the flask as an almost black layer of velvety shine. It dissolved reversibly and without residue in distilled water. The yields determined in several trials are shown in Table 1.

The urinary melanin prepared by the above described method was made into a 40 mg per cent distilled water solution and the extinction was measured at 10 wave- lengths in an Uvifot ultraviolet spectrophotometer. The curve

showing the values $E = 10\ g \cdot \frac{10}{I}$ plotted against wave- length was compared with the light-absorption curve of the tyrosine-melanins formed during the oxidative desamination of tyrosine solutions (40 mg per cent, pH around 7) by hydrogen peroxide in the presence of copper (tyrosine 1 : copper 0.1 : hydrogen peroxide 10) [5]. The artificial tyrosine-melanins prepared under conditions simulating biological conditions as regards pH and temperature, showed light absorption that was in many respects similar to that of natural urinary melanin.

Table 1

Preparation of urinary melanin

A Urine ml	B solids		C Dialyzed water-soluble preparation		
	g	per cent A	g	per cent B	per cent A
530	23.4	4.4	1.64	7.0	0.31
1150	65.5	5.7	0.45	0.7	0.039
1000	58.3	5.8	0.36	0.6	0.036

It was particularly conspicuous that every examined curve showed peaks at 510 and 564 mμ Bu' Lock [1] claims that from among the intermediates of the melanin polymerizate prepared from 5.6-dihydroxyindole by enzymatic oxidation those showing absorption maximum at 530 mμ are dimers, while the 540 mμ peak indicates trimers. It is beyond doubt that in the examined solution of the synthetic tyrosine-melanin the second and third members of the polymer chain were formed in the course of the synthesis of the macro-molecule. But it is unclear whether in the solution of the urinary melanin the same compounds are intermediates of synthesis or breakdown.

Moreover, the question emerged whether the excellent solubility of the urinary melanin prepared by the above method was due to the considerable proportion of dimers and trimers it contained, resulting in a low relative molecular weight, or to the lyophilic properties of the protein conjugated with the pigment. To solve the problem, we subjected the melanin preparation to HCl hydrolysis, dried it *in vacuo*, redissolved it in distilled water and, after that, examined it by paper chromatography (butanol : acetic acid : water 4 : 1 : 5, Schleicher-Schüll 2043*b* paper, onedimensional descending technique). The numerous unmistakable spots appearing on development with ninhydron proved that the urinary melanin contained protein. In the hydrolyzate, soot-like insoluble granules were found: they represented macro-molecular melanin of "rest-pigment" nature, deprived by acid hydrolysis of the protein which

ensures solubility. The liquid phase of the hydrolyzate was dark brown; the preparation had, thus, also components of low molecular weight, soluble even without protein.

For the sake of comparison the chromatograms of a few melanin preparations, those of their hydrolyzates and one or two potential intermediates, developed by different reagents, are presented in the following.

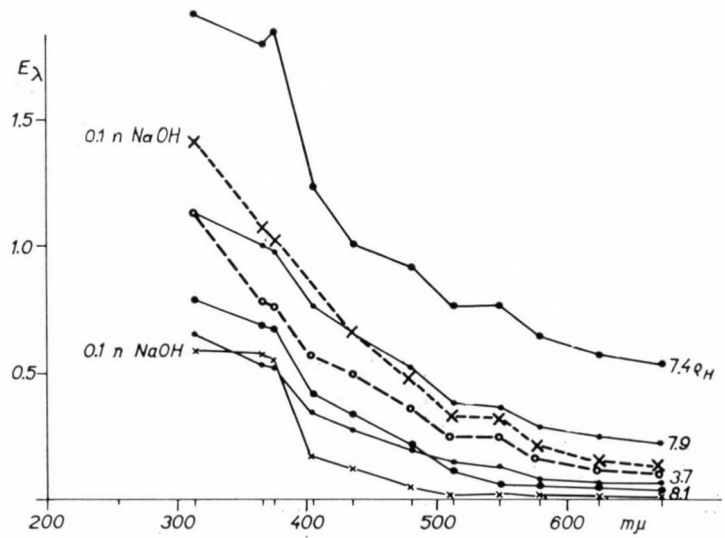

Fig. 2. Absorption curves of melanin solutions (40 mg/100 ml) made up with different buffers or 0.1 n sodium hydroxyde. — — — melanin from urine, ——— melanin from tyrosine

The chromatogram developed with ninhydrin shows many amino-acid spots of the melanin hydrolyzates (urinary melanin, neutral bovine-eye preparation). In the hydrolyzates of the bovine-eye melanins, boiled previously with acid of base, no amino acids were demonstrable. In the hydrolyzates containing amino acids the spots have the same positions, but owing to differences in concentration the chromatogram of the hydrolyzate of the eye melanin is two spots short because amino acids of the lowest concentrations did not appear. In agreement with the data published by GJESDAL [3], the amino acids were identified as glycine, alanine, valine, leucine, serine, cystine, asparatic acid, glutamic acid, arginine, phenylalanine and tyrosine.

After development with ammoniated silver solution (0.1 n $AgNO_3$: 5 n NH_4OH 1 : 1), three greyish blue spots, separable by running for longer periods of time could be visualized. Their R_f values were 0.07, 0.16 and 0.25. Three grey spots of similar Ri value (0.06, 0.24 and 0.30) were found by GHISLANDI [2] in the urine of patients with melanoma. He claimed them to have been produced by some melanotic substances. These spots are invisible without development, and we regard them as leuko-compounds produced in the course

of HCl hydrolysis of melanins that are capable of reducing silver, described to be characteristic from the point of view of the identification of melanin. The three characteristic products of melanin hydrolysis occurred not only in the urinary melanin but also in the hydrolyzate of the eye melanins (neutral, acid and alkaline).

The ferri-ferricyanide reagent, recommended for the demonstration of aromatic acids and phenols [4], produced a lively yellow spot at R_f 0.32 on the discoloured paper. The non-reducing compound giving the reaction is unknown. The reducing substances, for example DOPA, appeared as dark blue spots on the paper stained green.

We determined also the total N content of the melanin preparations compared in this study by the Kjeldahl method. The results are summarized in Table 2.

Table 2

Some properties of melanin preparations

	Total-N per cent	Solubility in distilled water	Protein content
Urinary melanin	6.6	+	+
Bovine-eye melanin, neutral	10.1	—	—
Bovine-eye melanin, acid	4.4	—	—
Bovine-eye melanin, alkaline	6.1	—	—
Tyrosine melanin, synthetic	5.3	+	—

The lowest nitrogen content was found in the acid bovine-eye melanin whose protein component had been removed to a maximum extent by hydrolysis. Likewise, almost all of the protein was removed from the alkaline bovine-eye melanin. The insolubility of these preparations is due to the absence of the lyophil protein component, and their properties are identical with those of the rest-pigment described by GJESDAHL [3]. No amino acid could be demonstrated in the chromatogram of their acid hydrolyzates.

The readily soluble urinary melanin contains much more total nitrogen, a phenomenon due to the protein conjugated to it which contains an average of 17 per cent N, as compared with the 4.4 per cent value of the restpigment. The highest total N values were found in the neutral bovine-eye melanin, not treated either with acid or base, so that this contained the highest concentration of protein. This is confirmed by the presence of the HCl hydrolyzates of the two latter preparations.

It is noteworthy that not all native melanin preparations containing protein are soluble in distilled water, or form suspensions stable in buffers of about pH 7. According to GJESDAHL [3], bovine-eye melanin produced by the

gentlest method, i.e. not treated with either acid or alkaline reagents, was not soluble either in water or in vegetable oil. On the other hand, the melanin preparation described above is reversibly soluble without residue even in distilled water and also 4 to 5 per-cent solutions for injection can be prepared of it.

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EIN WASSERLÖSLICHES MELANINPRÄPARAT

Zur Untersuchung der Biologie melanotischer Geschwülste benötigten Verfasser ein in neutralem wässerigem Substrat lösliches Melaninpräparat. Die Löslichkeit wird in erster Linie durch die an das Pigment gebundene Eiweißfraktion gewährleistet. Mit der Methode von Hohenadl und De Paola stellten Verfasser aus der Pigmentschicht des Rinderauges durch Auswaschen in destilliertem Wasser ein »neutrales« Melaninpräparat her, welches aber nicht löslich war. Aus dem Harn von Melanomkranken isolierten sie — in Ermangelung eines Lyophylisierapparates — nach Eintrocknung im Vakuumexsikkator und nachfolgender Dialyse die in der Lösung verbleibende Substanz. Dieses Melaninpräparat ist in destilliertem Wasser reversibel löslich. Es enthält 6,6% Gesamtstickstoff. Im salzsauren Hydrolysat des Präparates wurden papierchromatographisch mittels Ninhydrinreaktion 11 Aminosäuren, mit Hilfe von ammoniumhaltiger Silberlösung weitere drei Flecke und schließlich mit Hilfe von Ferriferrozyanid-Reagens noch ein unbekannter gelber Fleck nachgewiesen. Die Lichtabsorptionskurve des Harn-Melanins ist in vieler Beziehung der Kurve des aus Tyrosin hergestellten synthetischen Melanins ähnlich.

РАСТВОРИМЫЙ В ВОДЕ ПРЕПАРАТ МЕЛАНИНА

В целях исследования биологии меланотических опухолей авторы стремились получить меланин, растворимый в нейтральном водном субстрате. Растворимость обеспечивается прежде всего белковой фракцией, связанной с пигментом. По методу *Хохенадла* и *Де Паола* авторы из пигментовой оболочки глаза крупного рогатого скота вымыванием дистиллированной водой изготовляли «нейтральный» препарат меланина, который, однако, не растворился в воде. Из мочи больных меланомой, не имея лиофилизирующего

аппарата, авторы после высушки в вакуумэксикаторе и последующего диализа изолировали оставшееся в растворе вещество. Этот препарат меланина обратимо растворим в дестиллированной воде. Препарат содержит 6,6% общего азота. В его солянокислом гидролизате при помощи ингидриновой реакции на бумажной хроматограмме удалось выявить 11 аминокислот, при помощи раствора серебра с содержанием аммония — еще 3 пятна, а при помощи берлинской лазури — еще одно невыясненное желтое пятно. Кривая поглощения света меланином из мочи во многих отношениях подобна кривой синтетического меланина, полученного из тирозина.

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METABOLISM OF NICOTINE IN TOBACCO

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Synopsis

To provide a method for the investigation of metabolism of nicotine in tobacco a procedure has been elaborated for the separation of amino acids and nicotine on Dowex 2 ion exchange resin (OH^--form). By the use of this method it has been shown that nicotine-C^{14} fed to *Nicotiana tabacum* is almost completely broken down. Simultaneously C^{14}-labelled free amino acids are formed. The results suggest a close correlation between nicotine breakdown and nitrogen metabolism in tobacco.

Introduction

The physiological role of alkaloids in plants, including nicotins, is little understood. However, the old idea that the alkaloids are inert by-products of plant metabolism is no longer maintained [8, 10, 11]. Data are accumulating to show the important physiological role of nicotine. It has been found that the synthesis of nicotine takes place in the young meristematic tissues exhibiting a high metabolic activity [11, 14]. It is also important that a considerable amount of nicotine is contained by ripening and germinating tobacco seeds whereas no nicotine was found in resting seeds [6].

Recently, by taking advantage of tracer techniques, a large body of information has been obtained concerning the role of nicotine in metabolism. It has been shown that the infiltration of *Nicotiana glutinosa* with nicotine results in the formation of nornicotine due to oxidative demethylation of nicotine [2, 5, 16, 20]. The metabolic activity of the methyl group of nicotine is also indicated by the work of LEETE and BELL [9]. These authors observed the formation of radioactive choline after infiltration of the tobacco plant with nicotine labelled with C^{14} in the methyl group. Thus evidence was presented for the role of nicotine as a donor of methyl groups in plants.

Other workers succeeded in demonstrating the role of nicotine in the biosynthesis of nicotinic acid, another important metabolite in living tissues [4]. It is highly probable that an oxidative breakdown of pyrrolidine is involved in the reaction and that the pyridine ring of the alcaloid is the precursor of nicotinic acid.

The pyridin ring of some other compound can also derive from nicotine. Feeding of the tobacco plant with double labelled (C and N) nicotine resulted

in the formation of labelled nornicotine and anabasine [18]. Evidence was obtained that the pyridine ring of nicotine underwent no change during the reaction.

Further studies on the metabolism of C^{14}-labelled nicotine in plant tissues indicated that nicotine is broken down readily and the radioactivity deriving from it appears in a large variety of compounds [19]. Therefore, it is safe to conclude that nicotine takes an active part in plant metabolism and can not be regarded as an inert substance. As amino acids participate in the biosynthesis of nicotine it seemed possible that amino acids occur among the breakdown products of nicotine as well. The aim of the present paper was to test the validity of this hypothesis.

Materials and methods

Preparation of nicotine-C^{14}

In order to obtain labelled nicotine 4-month old *Nicotiana tabacum* plants were fed with Na-acetate-2-C^{14} via the root system. The precursor was added to the nutrient solution. After a 10-day treatment the plants were killed and the nicotine was isolated from the tissues according to standard methods [15]. Fifty per cent of the radioactivity of the nicotine synthetized from acetate-2-C^{14} is localized in the C_2 atom of the pyrrolidine ring and the rest in the pyridine ring [3]. The nicotine dipicrate obtained was dissolved in 1 *N* NaOH. MgO and NaCl was added to the solution and the nicotine was separated by steam distillation. Nicotine was determined in the distillation product by titration with 0.1 *N* H_2SO_4 in the presence of methyl red indicator. Part of the solution obtained was used for the determination of nicotine concentration by the use of the same method [15].

A standard solution containing 2 mg nicotine/ml was prepared for feeding experiments. The radioactivity of the standard solution was determined.

Method for the separation of nicotine and amino acids on Dowex 2 ion exchange resin (OH^--form)

A method for the separation of nicotine and amino acids was elaborated to make the study of eventual transformation of nicotine into amino acids possible. The usual method of isolating nicotine from alcaline media by steam distillation [15] proved unsatisfactory as the amino acids undergo a considerable decomposition under these conditions. Therefore, attempts were made for the separation of nicotine and amino acids by the use of an ion exchange resin (Dowex 2 in hydroxyl form). A 1 cm×20 cm column was prepared essentially according to PAVLOVSKAJA et al. [12]. Before preparing the column the resin (Dowex 2—4/20/50 mesh) was washed with bidistilled water to remove the

particles of undesirable size. In order to achieve an even distribution of the particles the resin suspension was poured into the column filled with bidistilled water to a height of 10—15 cm. First 0.5 l 10 per cent NaOH was passed through the column. The excess of NaOH was removed with H_2O. The resin was washed with water until a neutral reaction was reached. This was followed by washing the column with 1 l purified 2 *N* HCl. (Concentrated hydrochloric acid was purified by threefold boiling in the presence of $SnCl_2$. This treatment seemed important to remove substances giving reaction with ninhydrine.)

The excess of acid was removed by washing with bidistilled water until the test for Cl-ions with $AgNO_3$ proved negative. The resin was then transformed into the OH-form by treatment with 2 *N* NaOH. (The NaOH solution was always freshly prepared.) The excess of alcali was again removed by H_2O as described above and the column was ready for use.

In order to find out whether or not the resin is able to separate the amino acids and nicotine a number of amino acid + nicotine mixtures were tested.

The concentration of amino acids [7] and nicotine [15] was determined in control tobacco plants and standard amino acid + nicotine mixtures, corresponding to the natural levels of these substances, were prepared for the assays. The substances were dissolved in a final amount of 10—15 ml H_2O and this solution was put on the column. The resin was then washed with 1.5 l double distilled water. Under these conditions all the alcaline substances, sugars and non- electrolytes are released [13].

Nicotine was obtained by controlled elution. The end of elution was established by continuously testing the eluates for the presence of nicotine by adding silicotungstic acid to the solution [15]. By the end of elution the nicotine containing solution was acidified and evaporated to a small volume on a water bath. From the concentrated solution the alkaloid was precipitated with picric acid under cooling. In order to obtain pure preparations recrystallisation proved necessary. The final product was identified on the basis of crystal form, optical properties (absorption spectrum) and melting point of the dipicrates. The yield of nicotine obtained after passing through the column is shown in Table 1.

Table 1

Recovery of nicotine after passing through a column of Dowex 2 ion exchange resin (OH^--form)

Nicotine put on the column, mg	Nicotine obtained mg	Melting point of nicotine dipicrate	Recovery in %
40.0	39.7	217° C	99.2
45.0	44.0	217	98.0
50.0	49.4	218	98.8
55.0	54.8	217	99.6

As may be seen from Table 1 the nicotine passed through the column was almost entirely recovered. It was also important to find out whether or not the amino acids are quantitatively bound to the resin under the same conditions. This has been checked by washing the column with 0.5 l *N* HCl. The excess of HCl was removed from the eluate under vacuum at 36° C.

After the removal of HCl the pH of the solution was adjusted to 6.8. The concentration of amino acids was determined colorimetrically by adding ninhydrine to the eluate [7]. The results obtained are summarized in Table 2.

Table 2

Recovery of amino acids after passing through a column of Dowex 2 ion exchange resin (OH^--form)

Mixture No. 1			Mixture No. 2			Mixture No. 3		
Amino acids put on the column in μg	Amino acids eluted from the column		Amino acids put on the column in μg	Amino acids eluted from the column		Amino acids put on the column in μg	Amino acids eluted from the column	
	μg	%		μg	%		μg	%
211.8	186.4	88.0	232.0	227.3	98.0	29.9	29.3	98.0
424.0	381.6	90.0	464.0	459.4	99.0	59.8	59.3	99.1

Mixture No. 1: Lysine, histidine, asparagine
Mixture No. 2: Glycine, valine, serine, alanine, phenylalanine, proline, leucine, tyrosine and cysteine
Mixture No. 3: Asparatic and glutamic acids

As may be seen from Table 2 the acidic and neutral amino acids were almost completely recovered. By contrast, a considerable loss was experienced with the basic amino acids. On the average, however, a 95 per cent recovery was achieved.

It may be concluded from the results presented that the method applied is suitable for the quantitative separation of the amino acid fraction and nicotine.

For a further purification of amino acids, mainly to remove other contaminating acidic compounds, the solutions were passed through a column of Dowex 50 ion exchange resin in H^+-form.

A Dowex $50 \times 4/200/300$ mesh column was prepared as follows [17]: The small particles were removed from the resin by washing repeatedly with bidistilled water followed by sedimentation. The resin was then treated with a double volume of 1 *N* NaOH at 100° C for 16 hours. After this treatment a 1 cm $\times$ 15 cm column was prepared and the resin was washed with bidistilled water until neutral reaction was reached. The resin was transformed into H^+-form by treatment with 6 *N* HCl followed by washing with water to remove the Cl^- ions (eluate tested with $AgNO_3$). After this procedure the column was ready for use.

Results and discussion

Four-month old *N. tabacum* plants grown in the greenhouse were used for the experiments. The plants were fed with nutrient solution and 48 mg labelled nicotine (total activity 84 000 imp./min.). It has been shown that in about 48 hours practically all the nicotine was taken up by the plant roots. A 4-day incubation period was applied in the experiments. This length of incubation period was in line with the findings of other workers as well which indicates a marked breakdown of nicotine in four days [4, 9]. In the case of longer incubation periods the nicotine fed to the tobacco plant seemed to be protected against breakdown. This is explained by the above authors by the fact that during the long incubation the nicotine reaches the above-ground plants organs in which the breakdown of nicotine is much slower than in the root.

After the incubation period the plants were homogenized in mortar and extracted for 100 hours under constant stirring with a 20fold volume of 70 per cent ethanol. Under these conditions amino acids [1] and nicotine [15] go into solution. The extract was filtered through filter paper on a Büchner funnel. For the removal of lipids and pigments the filtrate was treated with an equal volume of petrol (low boiling point fractions) in a separatory funnel. In contrast to Tso and Jeffrey [19] no ether was used for the extraction of lipids as according to our experience this results in a 15 to 20 per cent loss of amino acids.

As shown by spectrophotometry the application of the above procedure resulted in a complete removal of chlorophyll from the test solution. Part of the carotenoids was also removed.

The defatted alcoholic extract was evaporated under vacuum at 36° C. A yellowish solution was obtained which contained a mixture of various organic substances.

For the separation of nicotine the mixture was passed through the Dowex column in the OH^--form as described above. The pH of the nicotine-containing solution obtained was adjusted to 4.0 with 0.1 N H_2SO_4 and the solution was evaporated on a water bath. From the concentrated solution nicotine was precipitated by picric acid according to a method described above [15]. The nicotine dipicrate obtained was used for the determination of alkaloid content and radioactivity.

The amino acid fraction separated in this way from nicotine, other basic compounds, and the nonelectrolyte fraction was further purified on Dowex 50 in the H^+-form [17]. The purified amino acids were used for quantitative determination and for the assay of radioactivity [7].

Nicotine contents and the radioactivity of nicotine isolated are shown in Table 3.

Table 3

Nicotine content and the radioactivity of nicotine

Nicotine content of control plants, mg	Nicotine content of experimental plants mg	Specific activity of nicotine imp./ min./in mmol	Total activity of nicotine imp./ min.	Recovery of radioactivity %
29.4	36.5	1.4×10^4	3120	3.7
28.5	30.5	1.4×10^4	2790	3.3

Average data from 2 parallel experiments.

As may be seen from Table 3 the nicotine content of experimental plants was very near to that of the controls. This means that about 84 per cent of the alcaloid fed to the plants (48 mg) was degraded. Therefore, the metabolism of nicotine in the tobacco plant is very intensive.

The results are in full agreement with those previously published on the intense breakdown of nicotine in ripening tobacco seeds [6].

The high metabolic activity of nicotine is also indicated by the low values of recovery of radioactivity which ranged between 3.3 and 3.7 per cent.

The results obtained are fully in line with the data of LEETE and BELL [9]. They also obtained only 5 to 8 per cent recovery in feeding *N. tabacum* plants with nicotine-C^{14}. By contrast, a 70 per cent recovery of labelling was reported by GRIFFITH et al. [4]. This is probably explained by the fact that another plant species *(N. glutionosa)* was used and the developmental stage of the plants was also different.

The decrease in specific acitivity of the nicotine isolated from the experimental plants (Table 3) as compared to the specific activity of nicotine fed to the tissues might be explained either by supposing that the nicotine taken up by the plants is diluted by endogenous nicotine or by the fact that the nicotine undergoes an active metabolism in the tissues.

The results obtained with the free amino acids are shown in Table 4.

Table 4

Amino acids and their radioactivity

Content of amino acids, μg/plant	Total activity of amino acids imp./min./plant
500	3000
590	2980

Average data from 2 experiments.

As may be seen from Table 4 the fraction of amino acids contains considerable radioactivity. This indicates that the breakdown products of nicotine might serve as precursors for the biosynthesis of amino acids.

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DIE UMWANDLUNG VON NIKOTIN IN DER TABAKPFLANZE

Um die Umwandlungsmöglichkeiten des Nikotins in der Tabakpflanze untersuchen zu können, wurde eine Ionenaustausch-Methode zur Trennung der Aminosäuren und Nikotin auf einer Dowex-2-Kolonne (OH^--Form) ausgearbeitet. Mit Hilfe der neuen Methode konnte Verfasserin nachweisen, dass das in die Pflanzengewebe eingeführte Nikotin-C^{14} fast vollständig metabolisiert wird. Gleichzeitig werden C^{14}-markierte freie Aminosäuren gebildet. Die erhaltenen Ergebnisse weisen darauf hin, dass zwischen Nikotin-Abbau und N-Stoffwechsel der Tabakpflanze eine enge Beziehung besteht.

ПРЕВРАЩЕНИЕ НИКОТИНА В РАСТЕНИИ ТАБАКА

При изучении процесса превращения никотина в растении табака разработан метод хроматографического разделения аминокислот и никотина на анионите Дауэкс 2 в OH^- форме. С помощью этого метода показано, что введение никотина — C^{-14} в N. **tabacum** приводит почти к полному его распаду и образованию свободных аминокислот C^{14}. Представленные результаты указывают на связь процесса распада никотина с азотистым обменом табачного растения.

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THE INITIAL PHASE OF CO_2 UPTAKE OF LEAVES

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Synopsis

The literature on photosynthesis regards the fixation of CO_2 by specific acceptors as the initial process of CO_2 uptake. The possibility that CO_2 is first dissolved is carefully discussed; in this case the association with the acceptor would be a process of secondary nature. The intracellular milieu is generally thought to be too acid to allow the dissolution of CO_2, which is an extremely mild anhydric acid. This misconception is seemingly supported by the current practice of removing CO_2 from carbonate-containing solutions by means of stronger acids. The present studies have shown that CO_2 is rather intensely soluble by the expressed sap of plants and by aqueous solutions of organic and inorganic acids. The principle that CO_2 uptake from the environment may begin with simple dissolution seems to be unobjectionable.

Introduction

In the literature on photosynthesis the question of how atmospherical CO_2 penetrates the cells has not been raised in a clear-cut way. As a rule, discussion begins with the acceptor problem and is focussed on the alternative of C_2 or C_5 acceptor. Irrespective of the nature of the acceptor, however, we suppose that CO_2 must first penetrate the cell and can combine with the acceptor only there.

The neglection of the initial part of CO_2 uptake is all the more surprising because the initial phase has been most thoroughly discussed, e.g. by MENGEL [3], in connection with the ion uptake of cells.

It seems likely that the lack of an opinion suggesting that dissolution should be the first step of CO_2 uptake is due to the difficulty of conceiving the absorption of the acid CO_2 by the cell sap, which is also acid. Table 1 shows the wide range of acidity of cell saps expressed from the leaves of various plants.

The cell sap obviously does not represent in every case the conditions prevailing inside the cell, where, separated from each other in a microheterogeneous system, quite different processes may take place side by side; yet it can be supposed that CO_2 when diffusing from the atmosphere into the cell cannot meet its acceptors unless it has passed over the hydrate cover of the cell wall mycelles and diffused to a certain distance in the cell sap being present in the cytoplasm. According to VEJLBY [4] the CO_2 acceptors are

Table 1

Acidity of the expressed sap of leaves (adapted from DRAWERT [1])

Plant	pH value	Method	Author
		high acidity	
Begonia semperflorens ...	1.3—1.6	?	RUHLAND—WETZEL 1926
Oxalis repens	1.9—2.2	H-electrode	ROGERS—SHIVE 1932
Rumex acetosella	2.5—2.7	H-electrode	ROGERS—SHIVE 1932
		medium acidity	
Bryophyllum calycinum ..	3.5—4.9	H-electrode	INGALLS—SHIVE 1931
Fagopyrum sagittatum	4.9—5.4	H-electrode	INGALLS—SHIVE 1931
Sedum weinbergii	4.5	Chinhydron electrode	DRAWERT 1948
Sempervivum glaucum	4.6	Chinhydron electrode	KESSLER 1935
		low acidity	
Zea mays	5.0—5.6	H-electrode	GUSTAFSON 1924
Lycopersicum esculentum ..	5.6—6.9	H-electrode	INGALLS—SHIVE 1931
Solanum tuberosum	5.8	colorimetry	WAGNER 1916
Triticum aestivum	5.9—6.2	H-electrode	DRAWERT 1948
Secale cereale	5.9—6.2	chinhydron-electrode	KEYSSNER 1931
Spinacia oleracea	6.2	glass half-cell	JÖRGENSEN 1939
Cucurbita maxima	6.4	H-electrode	GUSTAFSON 1924
Avena sativa	6.4	colorimetry	ARLAND 1924
Chenopodium vulvaria ...	6.5	chinhydron electrode	KEYSSNER 1931

localized in the chloroplast; such acceptors are accessible only through the cytoplasm and the cell sap layer surrounding the chloroplast. In many cases this interior environment is certainly much more acid than the acidity attainable by dissolving CO_2.

Since dissolution of CO_2 is not analogous to chemical reactions, it is not surprising that comparatively even mild organic acids are able to expell CO_2 from carbonates. The following series of experiment were performed in order to demonstrate whether or not simple dissolution can be the initial process of CO_2 uptake.

Material and methods

The absorption of chemically pure CO_2 gas by acid solutions, namely (1) by expressed saps of plants; (2) by solutions of various concentrations of acids taking part in the Krebs cycle and of other acids present in the plant cell; (3) by solutions of other organic acids; (4) by solutions of strong inorganic acids, was measured.

A special gas pipette [2] was filled with the solution to be tested. CO_2 was then introduced into the pipette and the volume of the CO_2 was calculated from the length of the gas column arising in the capillary tube of the apparatus. The gas and the solution were thoroughly mixed with a threaded nozzle and the amount of the gas absorbed was calculated from the shortening of the gas column.

Results

The experiments were carried out at the temperature of the summer season, 1963 (28—31° C). The data shown in Table 2 were read after mixing for a few minutes.

Table 2

Solubility of CO_2 in liquids of acid nature

Liquid	Dissolved gas CO_2 mm^3/1 ml liquid
Pressed-sap of *Sambucus nigra* leaf ...	approximately 10
Pressed-sap of *Tradescantia* spec.	approximately 40
Citric acid 25%	approximately 108
Citric acid 12.5%	approximately 134
Citric acid 6.3%	approximately 163
Citric acid 3.1%	approximately 210
Malic acid 0.1%	approximately 95
Aconitic acid 0.1%	approximately 205
Succinic acid saturated	approximately 175
Fumaric acid saturated	approximately 103
Ascorbic acid 0.1%	approximately 225
Malonic acid 0.1%	approximately 195
Tartaric acid 0.1%	approximately 175
Maleic acid 0.1%	approximately 230
Aspartic acid saturated	approximately 140
H_2SO_4 10%	approximately 115
H_2SO_4 5%	approximately 140

Discussion

The data presented in Table 2 are only valid under the given conditions. The cautions of the experiment were not strict: the amount of the introduced CO_2 and the rate of mixing were variable etc.; yet all the tested liquids actually absorbed CO_2.

One should not try to find stoichiometric relations in Table 2 for the very reason that the liquids were not saturated with CO_2. Thus, in fact, the expressed saps and the solutions of the organic and inorganic acids tested are able to absorb more CO_2 than shown by the figures.

Succinic, fumaric, and aspartic acids were used in saturated aqueous solutions. This fact does not mean high concentrations, for the water-solubility of these substances is poor. The pH values obtained with the METROHM apparatus were 1.5, 1.8 and 2.3, respectively.

Although our data are certainly objectionable from the analytical point of view, the fact that the solutions under study are able to absorb more CO_2 than shown in the Table supports our hypothesis instead of weakening it. Therefore, it is reasonable to regard dissolution as the initial process of CO_2 uptake.

We are of the opinion that atmospherical CO_2 readily meets the requirements of photosynthesis through the intercellular spaces of the leaf (on the average, 20 mg CO_2 per hour are fixed by 1 dm^2 of leaf surface), although a quite different dissolution equilibrium would be expected if calculated from the concentration of 0.03%. However, the system is not a statical one; there is no equilibrium between the gas and the liquid phase because gas equilibrium is gradually shifted towards dissolution by the photosynthetic CO_2 acceptors regenerating cyclically in the cells. Thus, a peculiar "steady state" is established.

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PARTIE INITIALE DE L'ABSORPTION DE CO_2 PAR LES FEUILLES

L'auteur attire l'attention sur le fait que, dans la litérature technique sur la photosynthèse, l'attachement à des accepteurs spécifiques est considéré comme le procès initial de l'absorption de CO_2. La question de la dissolution éventuelle de CO_2 est traitée avec précaution; dans ce cas, la combinasion avec l'accepteur ne serait que de nature secondaire. En général, le milieu intérieur de la cellule est beaucoup plus acidique que CO_2 qui est, en effet, un acide anhydrique extrèmement faible, la dissolution peut donc paraître impossible. Cette erreur est supportée par la pratique assez répandue de faire sortir CO_2 des solutions de carbonates à l'aide d'acides plus forts. Au cours de ses examens, l'auteur vient de constater que les sucs vacoulaires des plantes, ainsi que les solutions aqueuses des acides organiques et anorganiques dissolvent le CO_2 d'une manière assez intense. L'opinion, selon laquelle l'absorption de CO_2 de l'ambiance commence par une simple dissolution, ne se heurte pas á des obstacles de principe.

НАЧАЛЬНЫЙ ПРОЦЕСС ПОГЛОЩЕНИЯ CO_2 ЛИСТЬЯМИ

Автор обращает внимание на то, что в литературе по фотосинтезу начальным процессом поглощения CO_2 считают присоединение двуокиси углерода к специфическим акцепторам. Очень осторожно обращаются с вопросом, не имеет-ли здесь место растворение CO_2; в этом случае соединение ее с акцептором являлось бы уже процессом вторичного характера. Так как CO_2 представляет собой черезвычайно слабую англидро-кислоту, по сравнению с которой внутренняя среда клетки значительно более кислая, поэтому растворение может казаться невозможным. Это мнение поддерживается также и той распространенной практикой, согласно которой CO_2 освобождают из растворов карбонатов более сильными кислотами. В процессе своих исследований автор установил, что выжатый растительный сок, далее водные растворы органических и неорганических кислот довольно интенсивно растворяют CO_2. Следовательно, мнение, согласно которому поглощение CO_2 из окружающей среды начинается просто растворением, не встречается с принципиальным возражением.

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COMPARATIVE STUDIES ON DATURA STRAMONIUM AND ITS SYMBIOTIC MICRO-ORGANISM

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Synopsis

A micro-organism has been isolated from *Datura stramonium* seeds. It has been shown that the same pigment is contained by both the micro-organism and the *Datura* seeds. A qualitative and quantitative similarity between the amino acids of *Datura* seeds and those of the micro-organism isolated from the seeds has been demonstrated. Immunological investigations (agglutination, precipitation tests) revealed a marked similarity between the immunological properties of proteins isolated from *Datura* seeds and from the micro-organism respectively. The micro-organism contained a factor which exhibited a pharmacological and biological atropine-reaction. The factor isolated in crystalline form induced an atropine-like reaction in the eye of cat. All these results suggest a new form of symbiosis between the plant cell and the microorganism studied

Introduction

It has been shown in a previous communication that there is a close similarity between the pharmacological action of *Datura stramonium* and *Allium cepa* and a micro-organism isolated from the tissues of these higher plants [2]. The micro-organism isolated from *Datura stramonium* exerted on frog heart even after 50 passages an atropine-like effect. The similarity between the effects of the higher plant tissues investigated and the micro-organism isolated from them raises the question as to the nature of symbiotic relationship in this particular case. Are we facing a biochemically highly specific symbiosis or, as it has been postulated by some authors [12, 13], should the isolated micro-organisms be regarded as extracellular developmental stages of mitochondria? In the author's opinion this question can not be answered as yet.

The present studies were aimed at the elucidation of the chemical relationship between the *Datura* seeds and the isolated microorganism. Compounds exhibiting similar properties were looked for both in the seeds and in the micro-organism. The common occurrence in the host and micro-organism of a hemolytic factor and a fluorescent pigment was studied. The similarity of immunological properties and amino acid composition was also investigated.

Attempts were made to isolate in higher quantities an atropine-like substance from the micro-organism in order to carry out a test-reaction with the eye of cat which proved unsuccessful in former experiments probably due to

the low concentration of the compound to be tested. Also, pure substance was needed to start investigations on the elucidation of the chemical structure of the active agent.

Experimental

Hemolytic effect

In the course of studies on the biological properties of the micro-organism isolated from *Datura* seeds an intensive hemolytic effect was observed on blood-agar-plates. In order to test as to whether the hemolytic factor is present in *Datura* seeds as well the following experiment was carried out: 2 grams of *Datura* seeds were treated with 90 per cent alcohol for 5 minutes. The alcohol adsorbed to the surface of seeds was removed by burning and the seeds were homogenized in a sterile mortar in the presence of 5 ml sterile saline. The extract was centrifuged and subjected to ultrafiltration. Seven to eight drops of the filtrate were placed in the holes of the blood-agar plate. In about 12 hours total hemolysis was observed around the holes containing extracts from *Datura* seeds. The diameter of the hemolytic ring amounted to 10 to 12 mm. These experiments clearly show that both the *Datura* seeds and the micro-organism isolated from them contain a hemolytic substance.

Fluorescent analysis

It has been reported in a previous communication that both the *Datura* seeds and the micro-organism isolated from them exhibit green fluorescence under the fluorescence microscope [2].

Twenty grams of the micro-organism grown on Bouillon containing 3 per cent glucose were extracted with alcoholacetic acid mixture. The extract was evaporated and after the removal of alcohol alkalified with 10 per cent NH_4OH. The alcaline solution exhibited an intense blue-green coloration.

Extracts were prepared as described above from *Datura stramonium* seeds as well. The addition of NH_4OH also resulted in the formation of blue-green colour. As reported by Richter [cit. 1] "Der Samen des *Datura* und des *Hioscyamus niger* enthalten einen grün fluoreszierenden Körper, welcher in angesäuertem Wasser löslich ist und aus alkalischen Flüssigkeiten mit Amylalkohol extrahiert werden kann". On the basis of this report I have succeeded in isolating the blue-green pigment from the alkaline medium. The extracted pigment has a green fluorescence in UV light (Fig. 1). Thanks are due to Dr. J. Szőke for the preparation of spectrograms. The same compound could be extracted from the micro-organism isolated from *Datura* seeds.

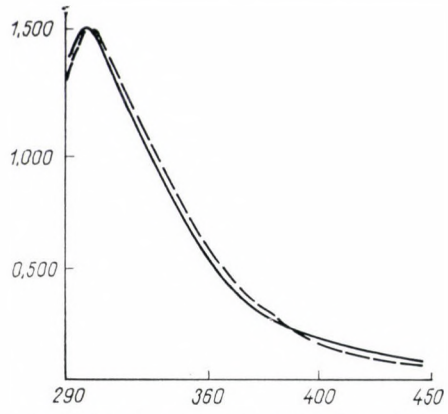

Fig. 1. Absorption spectrum of the pigment extracted from *Datura stramonium* seeds and from the micro-organism isolated from them. Absorption maxima at 298 mμ

Immunological studies

The above results prompted studies on the possible immunological relationship between the proteins of the Datura seeds and those of micro-organism isolated from them. The immunological methods are of a great value in establishing the nature of relationship between various organisms [7, 8].

Rabbits were injected with a sterile ultrafiltered extract of *Datura* seeds (5 grams of seeds homogenized in 25 ml saline). Simultaneously control blood samples of the same rabbits were tested for possible unspecific precipitation with the *Datura* extracts. These tests gave negative results. The injections were then repeated with increasing doses (0.5, 1.0, 1.5, etc. ml) on every 3rd day.

At the same time other rabbits were injected with samples of a 18-hour Bouillon-culture of the micro-organism. The control test for unspecific reaction was carried out with the micro-organism as well.

After the 8th injection the rabbits were killed and their serum was used for agglutination tests. The micro-organisms isolated from *Datura* seeds agglutinated the sera of rabbits injected with seed extracts. Agglutination was obtained up to a dilution of 1 : 1280.

Precipitation reaction was also carried out with the immune serum (diluted to 1 : 3) and the ultrafiltered suspension. In about 10 to 20 minutes strong precipitation ring was obtained. *E. coli* and *P. pyocyaneus* gave no precipitation reaction with suspensions of *Datura* seeds.

The results of serological studies clearly indicate that there is a specific relationship between the Datura sedes and the micro-organism isolated from them.

The immunological relationship described above is very similar to that described by LEINER [5, 6]. He has found that the serum of rabbits injected with *Pelomyxa* agglutinates a micro-organism isolated from *Pelomyxa*.

Amino acid composition

The amino acid composition of *Datura* seeds and of the micro-organism isolated from them was also investigated. The method of SZÁRA [14] was applied. The homogenates of seeds and the micro-organism to be tested were treated with 20 per cent HCl for 2 hours at 120° C in autoclave. The material was then dried by air stream on a water bath. The dry hydrolysate was taken up in 1 ml distilled water. The water was evaporated again to dryness. This procedure was repeated twice and the rest of the hydrolysate was finally taken

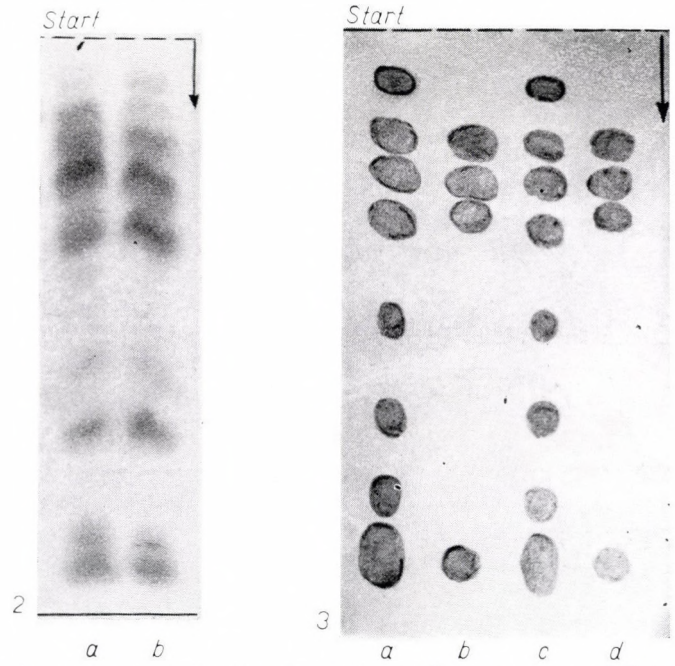

Fig. 2. Paper chromatograms showing the amino acid composition of *Datura stramonium* seeds (*a*) and that of the micro-organism isolated from them (*b*)

Fig. 3. Paper chromatograms showing the amino acid composition of *Datura* seeds (*a*), of *Allium cepa* (*b*) and of microorganisms isolated from *Datura* seeds (*c*) or from *Allium cepa* (*d*)

up in 2—3 ml H_2O. The solution was neutralized with silver oxide, followed by filtration. The filtrate was again evaporated and the residue was taken up in water. The hydrolysate was subjected to paper chromatography. Acetic acid—butanol—H_2O solvent mixture (1 : 4 : 5) and Macherey—619 filter paper was used throughout the experiments.

As may be seen in Fig. 2, the amino acid spectra of the hydrolysate from *Datura* seeds and the investigated micro-organism are identical.

Other micro-organisms were also investigated as to their amino acid composition *(E. coli, Pyocyaneus, Staphylococcus aureus)*. Comparative studies indicated that they have an entirely different amino acid spectrum. Similarly

it may be seen from Fig. 3 that the amino acid spectrum of the micro-organism isolated from Allium cepa and that of the mircoorganism isolated from *Datura* seeds is totally different. By contrast, there is a close relationship between the amino acid spectrum of the microorganism derived from *A. cepa* and the amino acid composition of *A. cepa* itself.

Pharmacological studies

It has been reported in a previous paper that the micro-organism isolated from *Datura* seeds, even after a 50 fold passage, exerts an atropine-like, acetylcholine inhibiting effect on the isolated frog heart [2]. By contrast, efforts to induce an opening of the pupil failed to give conclusive results. This could be explained by the low concentration of the active substance.

Attempts were made to produce the active ingredient in a sufficiently high concentration:

Mass-culture was prepared according to RÉDEY and KELEMEN [11]. 3000 ml of a 3 per cent Glucose-Bouillon medium (pH 7.2) was inoculated with the micro-organism isolated from *Datura* seeds. The culture was preincubated at 37° C for 12 hours in a thermostat. Further multiplication of the bacteria took place in a water-bath under aeration and constant stirring. The pH of the culture was checked during incubation and adjusted to the original value with 5 per cent NH_4OH. After incubation the bacteria were centrifuged. 10—15 g (wet wt) of bacterial cells were obtained.

Attempts were made for the extraction of the active agent from the bacteria by the method of STASS [cit. 1]. The bacterial cells were boiled for 4 to 5 hours in alcohol acidified with acetic acid. The cells were filtered off and the filtrate was subjected to distillation. The residue obtained was diluted with water. The oil layer observed on the surface was removed. The solution was made alcaline with 5 per cent NH_4OH and shaken out with chloroform. The chloroform was evaporated and the residue taken up in water. The solution gave a positive reaction (orange red colour) with the Draggendorf alkaloid reagent and exhibited an acetylcholine inhibiting effect in tests with frog heart. Treatment of cat eye with 1 ml of the solution resulted in 15 minutes in a maximal opening of the pupil. The effect lasted for 24 hours, and was easy to reproduce with the active ingredient repeatedly isolated from new cultures. The atropine-like effect of the substance is shown in Fig. 4.

I have succeeded in isolating the active substance in crystalline form. 28 grams of dried cells of the micro-organism served as starting material. The method proposed by KLOSA [3] for the extraction of atropine was applied. The dried cells were boiled for 8 hours in 1000 ml alcohol acidified with acetic acid. After extraction the alcohol was evaporated and the residue was taken up in water. The oil layer was removed from the surface of the solution which was shaken out several times with chloroform. The chloroform was evaporated

and the residue was taken up in warm alcohol. The cooling of alcohol resulted in the precipitation of a crystalline substance. After 10fold recrystallization from alcohol 300 mg pure, white, crystalline substance was obtained which exhibited an atropine-like action in tests with cat eye.

Fig. 4. Atropine-like effect on cat eye of the active substance isolated from the symbiotic micro-organism of *Datura stramonium*

Discussion

It has been shown that a symbiotic micro-organism isolated from seeds of *Datura stramonium* produces the same pigment as the *Datura* seeds. In addition, a marked similarity was found in the amino acid composition, immunological and pharmacological properties of the micro-organism and the *Datura* seeds.

The production by the micro-organism of a substance exhibiting atropine-like action is of a particular interest and suggests a close relationship between the host and the symbiotic micro-organism. It is clear from the results presented that the characteristic biochemical properties of the micro-organism, in particular the production of the atropine-like substance, are maintained after its isolation in pure culture.

A number of data ara available as to the biochemical effect of symbiotic micro-organisms on their animal and plant hosts [3, 5, 8, 9]. It might be assumed that the micro-organism greatly influences the physiological properties of the host.

It is quite probable that the relationship between *Datura* seeds and the micro-organism isolated from them represents a new form of symbiosis which has been unknown so far.

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VERGLEICHENDE UNTERSUCHUNGEN ÜBER DATURA STRAMONIUM UND SEINEN SYMBIOTISCHEN MIKROORGANISMUS

Es gelang in dem aus dem Daturasamen isolierten Mikroorganismus denselben fluoreszierenden Farbstoff nachzuweisen, den der Daturasamen enthält. Es konnte die qualitative und quantitative Ähnlichkeit der Aminosäuren des Daturasamens und des aus demselben isolierten Mikroorganismus festgestellt werden. Auf Grund von immunbiologischen Untersuchungen (Agglutination, Präzipitation) kann weiterhin eine weitgehende Identität der immunbiologischen Eigenschaften zwischen dem Daturasamen und dem aus ihm isolierten Mikroorganismus festgestellt werden. Aus grösseren Mengen des Mikroorganismus, die wir aus dem Daturasamen isolierten, konnte nach dem Atropinreaktionsverfahren eine pharmakologisch und biologisch atropinähnliche Wirkung nachgewiesen werden. Aus 28 g trockenem Mikroorganismus ist es gelungen 0,3 g kristallinischen, reinen Stoff zu gewinnen, welcher auf das Katzenauge eine atropinartige Wirkung ausübt. Alle diesen Umstände weisen auf eine zwischen der Pflanzenzelle und dem Mikroorganismus bestehende neue Form der Symbiose, über die in der Biologie bis jetzt keine Angaben bekannt waren.

СРАВНИТЕЛЬНЫЕ ИССЛЕДОВАНИЯ С DATURA STRAMONIUM И ЕЕ СИМБИОТИЧЕСКИМИ МИКРООРГАНИЗМАМИ

В микроорганизмах, изолированных из семян дурмана обыкновенного, авторам удалось обнаружить то-же самое флюоресцирующее красящее вещество, которое содержат и семена дурмана. Установили далее качественное и количественное сходство аминокислот в семенах дурмана и в изолированных из семян микроорганизмах. На основании

иммунобиологических исследований (агглютинация, преципитация) установили дальнейшую далекоидущую идентичность между иммунобиологическими особенностями семян дурмана и изолированных из них микроорганизмов. Из большого количества микроорганизмов, изолированных из семян дурмана, на основании атропиновой реакции обнаружили атропиноподобное действие, проявляющееся как в фармакологическом, так и в биологическом отношении. Из 28 г сухой массы микроорганизмов получили 0,3 г кристаллического, чистого вещества, которое в глазах кошки вызвало атропиноподобное действие. Все эти факты свидетельствуют о наличии новой формы симбиоза между растительными клетками и микроорганизмами, о которой до сих пор в биологии не было известно.

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HISTOCHEMICAL STUDIES ON GOMORI-POSITIVE SUBSTANCES

I. EXAMINATION OF THE GOMORI-POSITIVE SUBSTANCE IN THE ENDOLYMPHATIC SAC OF THE RAT

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Synopsis

The homogeneous (Gomori-positive) substance filling the endolymphatic sac of the rat gives positive reactions with methods for the demonstration of acid mucopolysaccharides, with the PAS-reaction, and with reactions demonstrating protein. In the authors opinion, the material of the sac is composed of an acid mucopolysaccharide and mucoprotein. It is supposed that the Gomori-positivity corresponds with the acid mucopolysaccharide. The Gomori-positive, homogeneous substance accumulated in the distal part of the endolymphatic duct is histochemically identical with the material found in the endolymphatic sac.

Introduction

BARGMANN [1] and GABE [5] observed that the hypothalamic-hypophyseal neurosecretion was electively stained by Gomori's chromealum haematoxylin [7] and paraldehyde-fuchsin [8]. The enormous volume of work done since then by means of the two methods has led to the coining of the term "Gomori-positive", which means that the substances in question stain equally well with chrome haematoxylin and with paraldehyde-fuchsin.

The two dyes can, however, be used for more purposes than the demonstration of neurosecretion. After previous permanganate-sulphuric acid oxidation, they stain also the intestinal cuticle, reticular tissue; without oxidation they stain elastin, hyaline cartilage, the granules of mast cells, etc. [9, 13, 18]. It is likely that both basic dyes, when used after oxidation, combine mainly with the acid groups containing sulphur, formed in the course of oxidation; and in the absence of oxidation with the acid groups originally present in the tissues, mostly with acid mucopolysaccharides [2, 3, 6, 11, 18]. According to RODECK [17], Gomori-positivity demonstrates the sulpho (SO_3H^-) groups, while KONEČNY and PLIČKA [11] claim that the dye may combine not only with the SO_3H-group but also with the SO_2H^-, COH^-, OSO_3H^-, and possibly also with other acid groups ($COOH^-$). Thus, the Gomori-positivity alone reveals nothing but the chemical group to which the substance in question belongs.

The present investigations were undertaken to analyze various Gomori-positive substances, first of all those occuring in the nervous system. This paper deals with studies concerning the Gomori-positive substance accumulated in

the endolymphatic sac whose histochemical nature has not yet been discussed in the literature.

The Gomori-positive substance being in the endolymphatic sac has been described by VIGH and co-workers [21]. The lumen of the endolymphatic sac, from the lower species up to man, contains a homogeneous substance which stains well with chrome haematoxylin and paraldehyde-fuchsin. In some species the material is abundant; in the axolotl, for example, the Gomori-positive substance accumulated in the voluminous endolymphatic sacs makes up about one-fourth of the total brain mass. The substance is presumably secreted by the epithelial cells of the membranous labyrinth [21].

The results of the present investigations have already been published in a short abstract [20].

Material and method

Newborn and adult rats were used. The animals were killed by decapitation, the petrous bone was removed together with the adjacent brain tissue and fixed in Bouin, Helly, 10 per-cent formalin or in 4 per-cent neutral Ca-formol. The adult animals were subjected to about six hours, the newborns to about two hours of electrolytic decalcination by the method of KULENKAMPFF [12]. A mixture of 3 per-cent HCL and 10 per-cent formic acid was used for this purpose. One part of the new born rats were not electrolytically decalcinated but in Bouin fixative for 24 hours. The specimens for lipid tests were embedded in nonex, all others in paraffin. The sections were 7 to 12 microns thick.

A total of 39 labyrinths was studied. The staining methods and reactions employed were:

Gomori's chrome haematoxylin staining, as modified by BARGMANN [1], with and without oxidation; paraldehyde-fuchsin staining according to GABE [5] with and without oxidation, after fixation by Bouin's method.

PAS reaction with and without digestion (saliva), after Helly, Ca-formol, and Bouin fixation. The Schiff reagent was prepared according to DETOMASI [14].

Trypaflavine reaction for the demonstration of mucopolysaccharides containing sulphur, according to TAKEUCHI [19], after Helly and Ca-formol fixation. In the reaction, an M/20 aqueous acriflavine solution was used for 30 minutes and 1 hour, respectively.

Methylen blue extinction (MBE) for the estimation of the degree of basophilia, after Bouin fixation. Methylen blue was dissolved in Michaelis veronal-acetate buffer at pH 8.1; 7; 5.3; 4.1; 3.7; 2.3 in a concentration of 0.0005 *M*.

Toluidine blue for the demonstration of metachromasia, with 0.5 and 0.1 per-cent aqueous dye solution [14], after Helly fixation. Some of these sections were mounted with glycerol-gelatine, others were passed through tertiary butanol and xylene, and mounted with DPX. Further, a toluidine blue (TB) staining was carried out at various pH. 0.1 per-cent toluidine blue in Michaelis's veronal-acetate buffer at pH 6.5; 3.8; 2.8; 2.1 was used for 5 to 20 minutes after Bouin fixation. Digestion with hyaluronidase ("Hyason" from N. V. Organon, Netherlands, 30 E/ml 0.1 *M* phosphate buffer at pH 6.0) was followed by staining with 0.5 per-cent aqueous or 0.1 per-cent toluidine blue in Michaelis buffer at pH 3.8.

Astra blue staining for the demonstration of acid mucopolysaccharides. The dye was prepared according to PIOCH [15]; staining after Bouin fixation lasted 3 minutes.

Coupled tetrazonium reaction with stable diazonium salts, as a general protein reaction [16]. Fixation with neutral Ca-formol and Bouin's fixative. Ninhydrin-Schiff method for protein-bound NH_2 groups after Bouin fixation, and demonstration of SS- and SH-groups by the DDD method according to BARRNETT and SELIGMAN [14], after Ca-formol and Bouin fixative. Demonstration of tyrosine according to LILLIE [14] after Bouin fixation.

Nucleic acids were examined by the Feulgen and methyl green-pyronine reactions [10], formol fixation was used.

To detect lipids, Ca-formol fixation was followed by staining with Oil Red O and Sudan Black B [14].

Results

Results obtained by the staining methods and histochemical reactions are shown in Table 1.

Table 1

Results obtained by the staining methods and histochemical reactions

Stain or reaction	Fixation	Reaction of the substance contained in the endolymphatic sac
1. Chrome haematoxylin with oxidation	Bouin	strongly positive
2. Chrome haematoxylin without oxidation	Bouin	strongly positive
3. Paraldehyde-fuchsin with oxidation	Bouin	strongly positive
4. Paraldehyde-fuchsin without oxidation	Bouin	strongly positive
5. PAS reaction	Helly, Ca-formol	strongly positive
6. PAS reaction after digestion with saliva	Ca-formol, Bouin	strongly positive
7. Trypaflavine	Helly, Ca-formol	strongly positive orange-colored reaction
8. MBE	Bouin	strong basophilia at pH 3.7
9. Toluidine blue	Helly	purple metachromasia
10. TB at various pH	Bouin	purple metachromasia at pH 3.8
11. Toluidine blue with hyaluronidase digestion	Helly, Bouin	purple metachromasia
12. Astra blue	Bouin	positive
13. Tetrazonium reaction	Ca-formol, Bouin	positive
14. Feulgen's reaction	formol (10 per-cent)	negative
15. Methylgreen pyronine	formol (10 per-cent)	negative
16. Ninhydrin-Schiff for protein-bound NH_2	Bouin	positive
17. DDD reaction for SH groups	Ca-formol, Bouin	weakly positive, pink reaction
18. DDD reaction for SS groups	Ca-formol, Bouin	negative
19. Test for tyrosine	Bouin	negative
20. Sudan Black B	Ca-formol	negative
21. Oil Red O	Ca-formol	negative

The homogeneous substance accumulated in the lumen of the endolymphatic sac reacted intensively with both chrome haematoxylin and paraldehyde-fuchsin (Fig. 1). The staining remained even if no previous oxidation had been employed.

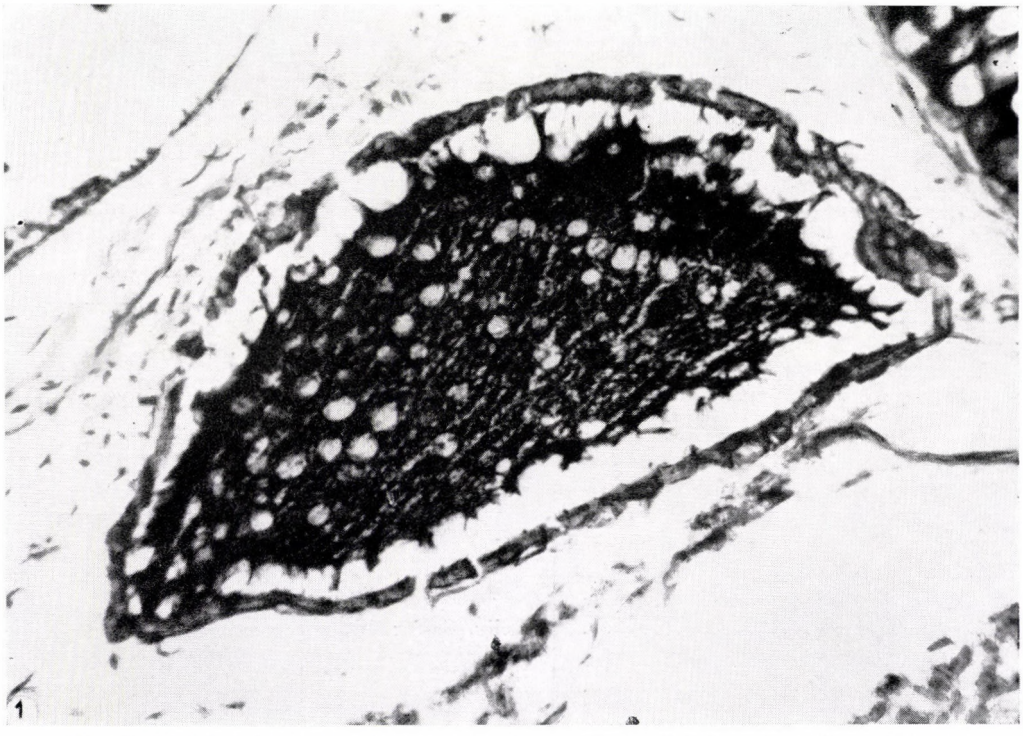

Fig. 1. Endolymphatic sac of the rat, its lumen filled with Gomori-positive substance

The substance in the sac gave an intensive PAS reaction (Fig. 2). Previous digestion with saliva did not diminish the intensity of the reaction.

The material stained orange yellow with trypaflavine, in an intensity similar to that shown by hyaline cartilage (Fig. 3).

With methylen blue extinction the most strong degree of basophilia was seen at pH 3.7.

Staining with toluidine blue at pH 3.8 resulted in purple metachromasia. The metachromatic staining remained after 18 and 23 hours of digestion with hyaluronidase.

With Astra blue, the substance in the endolymphatic sac gave a positive sky-blue staining.

The tetrazonium and ninhydrin-Schiff reaction was positive in the endolymphatic sac and in the cytoplasm of the epithelial cells composing the wall of the sac (Fig. 4).

The substance did not stain with the Feulgen reaction, nor with methyl-green-pyronine.

The DDD reaction detected no SS-groups, and it was only a pale pink colour that was obtained in the test for SH-groups. The test for tyrosine yielded likewise negative results.

The material of the sac stained neither with Sudan Black B, nor with Oil Red O.

Gomori-positive substance was found not only in the lumen of the endolymphatic sac but also in that of the endolymphatic duct (Fig. 5). The material

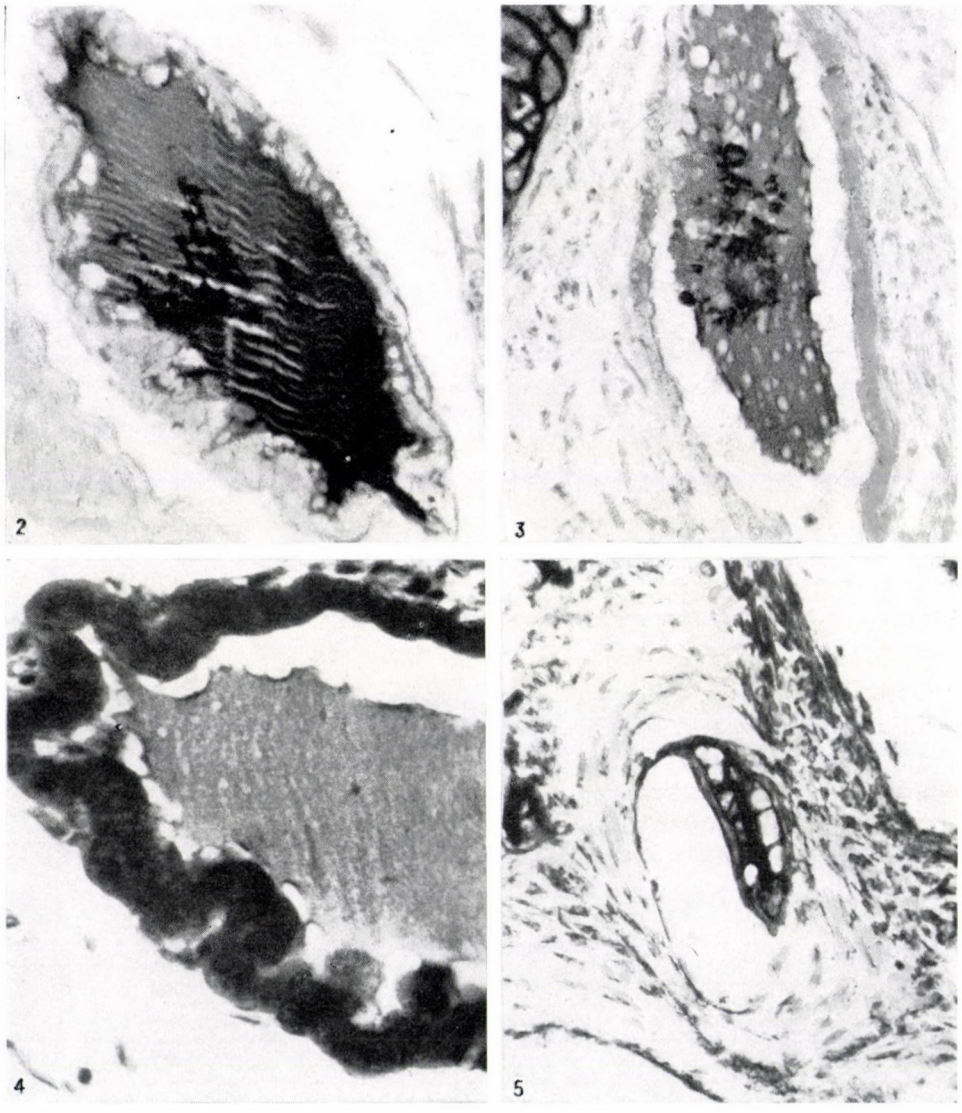

Fig. 2. Endolymphatic sac, PAS reaction, *Fig. 3.* Endolymphatic sac stained with trypoflavine. In the upper left corner, detail of a positively stained hyaline cartilage is visible. *Fig. 4.* Endolymphatic sac of the rat. Tetrazonium reaction. Marked positivity in the cells composing the wall. *Fig. 5.* Endolymphatic duct of the rat stained with paraldehyde-fuchsin. Note Gomori-positive homogeneous substance in the lumen

in the duct gave the same reactions as that in the sac. The amount of the substance was very small at the beginning of the duct and increased toward the sac.

No difference was found in these studies between the new born and adult animals as regards the quality of the endolymphatic material.

Discussion

The strongly positive reaction obtained with Gomori's chrome haematoxylin and paraldehyde-fuchsin even without oxidation, indicates that the substance contains numerous acid groups. At the same time, staining according to Gomori separates clearly the material of the sac from the neurosecretion, the latter being positive only after oxidation [1, 5].

The methylen blue extinction below pH 4, the metachromasia on staining with toluidine blue, further the negative result of the Feulgen reaction and the staining with methylgreen-pyronine point to the presence of acid mucopolysaccharides. The same is indicated by the positive staining with Astra blue. The orange yellow colour obtained by staining with trypaflavine suggests, according to TAKEUCHI, that the substance is composed of mucopolysaccharides containing sulphur.

There was no difference in the intensity of the PAS reaction with and without digestion. This indicates that the substance contains no glycogen, and that it seems to be perhaps composed of neutral mucopolysaccharides, mucoprotein or glycolipid. The failure of the stains Oil Red O and Sudan Black B to stain the material in the sac rules out the possibility that the examined substance possesses glycolipid properties.

The tetrazonium reaction was positive. This result was interpreted as a general indication of proteins. According to RAPPAY and PÓSALAKY this reaction is positive not only at the presence of histidine, tyrosine and tryptophan, as described in the literature, but of cysteine, dioxyphenylalanine and diiodotyrosine as well [16]. The tests for tyrosine yielded negative results, nor were disulphide linkages demonstrable in the substance. The positive sulphhydril reaction, however, indicates cysteine containing protein and the result of the ninhydrin- Schiff method demonstrates the presence of protein-bound NH_2 groups.

So, the strong positivity of the PAS- and of the protein reactions may indicate a mucoprotein. On the basis of our results we suppose that the endolymphatic sac contains two materials, probably a sulphated, acid mucopolysaccharide and a mucoprotein. Finally we think that between the two mentioned materials the acid mucopolysaccharide corresponds with the Gomori-positive substance since the material of the endolymphatic sac shows Gomori-positivity without oxidation. In such a case the Gomori stains combine with the acid groups originally present in a material, mostly with the acid mucopolysaccharides [2, 3, 6, 11, 18].

There was no difference in the reactions between the substance in the sac and in the distal part of the endolymphatic duct.

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HISTOCHEMISCHE STUDIEN GOMORI-POSITIVER SUBSTANZEN

I. Untersuchung des Gomori-positiven Materials im Saccus endolymphaticus der Ratte

Das homogene (Gomori-positive) Material, das den Saccus endolymphaticus der Ratte ausfüllt, gibt positive Reaktionen mit Methoden zum Nachweis saurer Mucopolysaccharide, mit der PAS-Reaktion, sowie mit Eiweiss nachweisenden Verfahren. Nach Meinung der Autoren ist das Material des Saccus zusammengesetzt, es besteht aus einem sauren Mucopolysaccharid und einem Mucoproteid. Es ist anzunehmen, dass das saure Mucopolysaccharid für die Gomoripositivität verantwortlich ist. Die Gomori-positive, homogene Substanz, die sich im distalen Teil des Ductus endolymphaticus anhäuft, entspricht histochemisch dem Material des Saccus endolymphaticus.

ГИСТОЛОГИЧЕСКОЕ ИССЛЕДОВАНИЕ ГОМОРИ-ПОЛОЖИТЕЛЬНЫХ ВЕЩЕСТВ

I. Гомори-положительное вещество эндолимфатического мешка у крыс

Гистохимическими методами показано, что (Гомори-положительное) вещество, заполняющее эндолимфатический мешоку крыс дает положительные реакции на белки, кислые мукополисахариды и дает положительную ШИК-реакцию. Авторы считают, что эти вещества состоят из кислых мукополисахаридов и мукопротеинов. Очевидно, Гомори-положительность зависит от кислых мукополисахаридов. Однородное Гомори-положительное содержание эндолимфатического потока является идентичным с гистохимической точки зрения с веществом эндолимфатического мешка.

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THE IMMUNOLOGICAL COMPETENCE OF THE THYMUS AND SPLEEN IN NEWBORN AND ADULT RAT

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Synopsis

The authors have studied the immunological competence of the newborn thymus and spleen, and that of the adult thymus and spleen in the rat. It has been found, in accordance with the evidence reported by Miller that the newborn thymus possesses prominent immunological potencies, which by the time adult age is reached are transferred to the spleen and lymph nodes; meanwhile, the thymus loses these properties to a great extent. Phylo- and ontogenetical causes are discussed.

Introduction

Miller reported in 1961 that in the mouse the spleen and lymph nodes would develop poorly in case thymectomy was performed in newborn age [18]. Immunity diminishes and at the same time the tolerance to heterologous (foreign) transplants increases. According to Miller in embryonic age the thymus contains immunologically competent cells, which pass over into the various lymphatic tissues after that age [18, 19]. His results have been confirmed by Jankovic and co-workers [1, 16, 27] in the rat, by Sherman and Dameshek [23] in the hamster; many other experiments were performed in this respect [5]. The suggestion itself that, as indicated by these experiments, the thymus might be considered to be the most important organ of immunity, gave rise to lively debates [20]. There is an abundance of research works contradicting that the thymus would be involved in immunity.

For example, Askonas and White [2] found no immune body producing activity of the thymus in guinea pigs examined by the use of fluorescent antigen, while Harris and Harris [15] have observed the same in the case of in vitro immunisation against dysentery. Azar [3] has examined the plasmacytopoietic activity of lymphatic tissue after immunisation and found that it was detectable in the lymph nodes and spleen, but not in the thymus. Thorbecke and Keuning [26], also studying tissue cultures, have found that immunisation evoked responses in various lymphatic tissues, but not in the thymus. According to Schelin and co-workers [22] plasma cells are not present normally in the thymus, and appear in it in small quantities only even in response to experimental interventions. Fich-

TELIUS [14] has a similar opinion. Some workers, *e.g.* CZAPLICKI [12, 13] go as far as to claim that a substance producing agammaglobulinaemia would be present in the thymus. Our earlier investigations, too [10, 11], indicated that even if plasma cells were formed in the thymus, this certainly is not so significant that the organ should be ascribed a key position in immune body production. Thus, it seems, if only in the light of the controversial evidence reported, that some incongruence exists between the potencies of the embryonic and newborn thymus and the actual capabilities of the gland in adult age. We have therefore carried out investigations to elucidate whether there is an identity in immune competence between the newborn and adult thymus and how this compares with the corresponding activities of the newborn and adult spleen.

Material and methods

The mortality due to "runt disease" was used as the test in our experiments [4]. Therefore we injected into 486 newborns of 62 rats within 24 hours from birth cell suspensions of homologous newborn spleen or thymus, and adult thymus and spleen suspensions respectively intraperitoneally, in 0.1 ml physiologic saline. Each newborn animal received 3 mg wet weight tissue. The transplants were obtained from 200 newborns of another 30 families, and from 36 adult rats. The rats came from our stock, considered to be a not-inbred, originally Wistar strain. After having received the injection of cell suspension, the newborns were put back to their own mothers. Only one kind of transplantation was carried out within one litter.

The runt disease manifested itself in retardation of growth (Fig. 1), roughing of the fur, weakness, diarrhoea then death. Considering that the test is evaluated on the basis of the mortality rate and time of death, the animals were not necropsied until after the age of 1 month, thus, we could not observe the internal changes produced by the runt disease. The animals surviving 30 days were necropsied, the organs were weighed, but we found no significant differences between the various groups, which is understandable, because the animals survived and thus the changes produced were not marked.

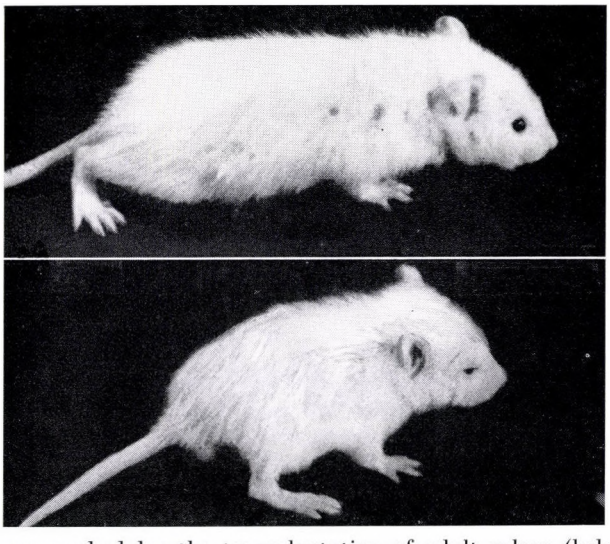

Fig. 1. Runt disease evoked by the transplantation of adult spleen (below). Above: well-developed control of similar age

Results and discussion

The results have been tabulated. Table 1 shows the number of animals in the group, then the mortality rate in per cent from 0 till 3 days. In our opinion in that period the animals perish not necessarily as a result of transplantation, but either because the mother does not let the offsprings suck, or the newborns fall victim to cannibalism, as it often occurs with rats. Therefore further results are expressed as the percentage of the animals still alive on the 3rd day and this is accepted as the realistic basis for further calculations.

As the description of the experimental procedure has revealed, we have reversed MILLER's technique [18] in our studies of the role of the thymus. While MILLER, and since then others [1, 16, 23, 27], extirpated the thymus and observed the effect, we transplanted the thymus and spleen at an age, at which the immunological tolerance is still existent in the rat [4]. Because of this, the newborn rat does not destroy the cells injected into it, on the contrary, they will presumably continue to grow in it. And if the transplant contains immunologically potent cells, they will produce immune bodies and will kill the immunologically immature or less mature host organism.

The ultimate results of the experiments indicate that if we take into consideration the number of survivors in the different groups, the destructive effects of the newborn thymus and adult spleen are clearly manifest, while the newborn spleen and adult thymus were by far less destructive. What does this imply? The only possible implication is that the immunological potencies, or immunologically potent cells of the embryonic thymus are transferred to the spleen and naturally, and even to a greater extent, to the lymph nodes by the time adult age is reached, when the thymus shows a great loss of these potencies. Thus, the evidence published by MILLER has been corroborated: the thymus of the newborn possesses in fact higher immunological potencies than the spleen of the newborn, even though with these experiments the absolute positivity and absolute negativity could not be proved, but we must add to this that all these cannot be applied to adult age. It seems that BURNET [5] is right: he does not claim at all that the thymus would take part in the production of the classic immune bodies, but thinks that the cells involved in systemic defence are descendants of the thymic cells, which attain full immunological potency in the spleen and lymph nodes. Thus, from the experiments of MILLER and the other authors referred to one in no way draws the conclusion that in every age the thymus plays a role in immune body production, or that it plays such a role at all; we may only claim that in a certain age, in the embryonic, the thymus is a carrier of immunological potencies, which it loses to a significant extent later. Thus, during the period between embryonic and adult age a migration of immunological potencies is observable, at the same time the different lymphatic organs become specialized. Therefore the lymphatic system

can never be considered to be essentially homogeneous [9, 10, 11], because at the time the thymus is highly potent immunologically, the spleen is less potent, whereas by the time the latter not only carries in it the potencies, but also realizes them, the thymus has lost them partially, as it is indicated by the present experiments, or totally, as it is indicated by earlier ones [10, 11]. On the other hand, by losing the general immunological potencies the thymus has attained its specialized function in the processes of tissue correlation, because as we have described earlier [7, 8] the adult thymus is much more capable of producing mast cells, than is the embryonic thymus.

Table 1

Group	Number of animals at start	Mortality rate (%) till 3rd day	Mortality rate (%)			Alive at 30 days per cent
			4 to 6 days	7 to 14 days	14 to 30 days	
Newborn thymus .	102	28.5	31.5*	31.5	5.5	31.5
Newborn spleen .	109	18.4	20.0	29.1	3.4	47.5
Adult thymus	99	35.1	11.2	31.2	8.1	49.5
Adult spleen	176	40.5	18.1	29.5	19.0*	33.4

* significant: $p < 1\ \%$

If we analyze the tabulated data, or represent them semi-diagrammatically (Fig. 2) it will seem that during the first three days the adult tissues

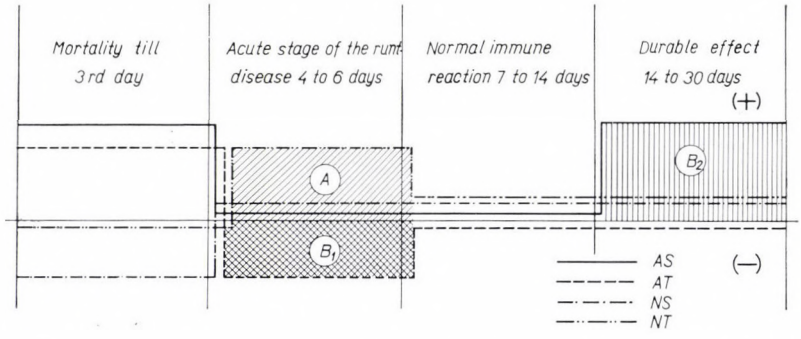

Fig. 2. Semi-diagrammatic representation of the effects of various transplantations

thymus and spleen, were more destructive than the embryonic ones. However, the destruction observed during that period was not taken into consideration in subsequent evaluation, let us therefore analyze the later points of time. In our opinion the period until the 4th—6th day is the acute stage of the runt disease, it is at that time that the actual destruction begins in our experiments in case immunologically potent cells are transplanted. In that phase the new-

born thymus was prominently destructive, while the adult thymus was hardly destructive. Significance analysis by the 4-field chi square method shows that the newborn thymus is significantly more active than the other organs. The next more or less arbitrarily selected period is that from the 7th till the 14th day. This period is considered to be the one in which immune bodies normally begin to appear and in the case of transplantation it is usually in this period that necrosis begins and the transplant is cast off. By that time the effects of the different splenic and thymic transplants show an equalisation, the mortality rates are closely similar, there are no significant differences. In the next period the adult spleen was prominently and significantly more destructive than the other organs. Thus, in our schematic figure the area marked A corresponds to the acute runt disease effect of the newborn thymus and this gives the difference from the newborn spleen, whereas the area B_1 shows the lack of such activity of the adult thymus, and the area B_2 indicates that the adult spleen has a durable effect. Thus, $B_1 + B_2$ gives the difference existing between the effects of the adult spleen and thymus. If we try to explain all these in the light of the clone selection theory, it may be stated that in the embryonic age it is actually in the thymus that we find the immunologically most potent cells, but in adult age they are present in smaller numbers, on the one hand, and, on the other, there is no ability to produce such cells for a long time (14 to 30 days), whereas the spleen possesses these properties. We shall return to this special ability of the spleen, in which lies primarily the difference between the newborn and adult spleen from the immunological point of view, in connexion with the statistical analysis.

The above evaluation of the chronological differences in the mortality rates cannot yield an absolutely realistic picture, because the duration of the periods has been determined arbitrarily. However, it seems to be rather unequivocal that, even though not in the rhythm of the effect, but in its fact the role of the newborn thymus is taken over from this point of view by the spleen in adult age, if we take into consideration the first month only. If we plot the dynamic curves of survival, the whole process can be followed up and the results will be similar to those outlined above (Fig. 3).

Evaluation according to days has been carried out on the basis of the "potencies of the effect", measured by the velocity of mortality (mortality/100 days). In the calculation 100 was divided by the days of death (subtracting in every case the first 4 days). The potency of the effect thus measured, i.e. the average velocity of the chain reaction causing death, can be considered to show normal, Gaussian distribution (Fig. 4). The average time of death was calculated by dividing 100 by the average velocity. Doubtlessly, this analysis although more precise in its method, has the shortcoming of ignoring the values before the 4th day, and considering only the sum of the values after the 30th day, and thus neither the averages, nor the distributions reflect the whole

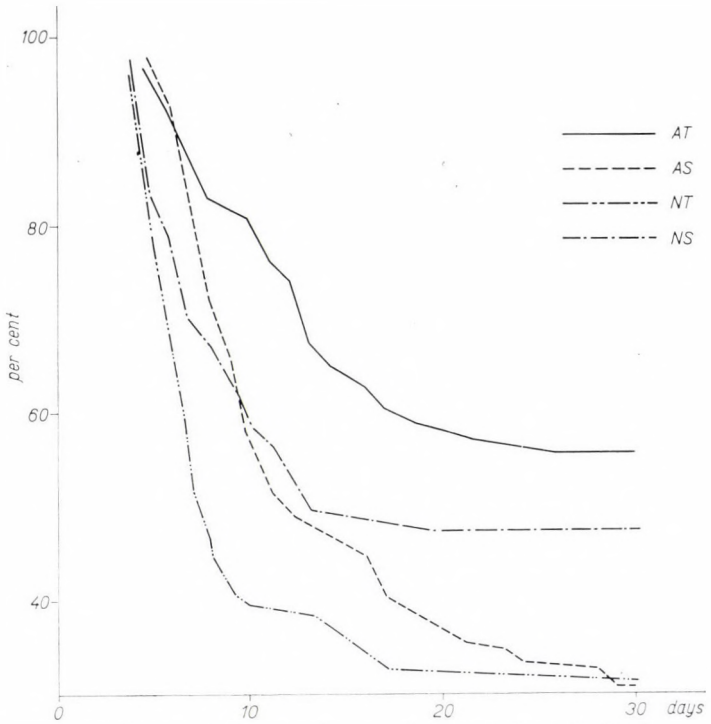

Fig. 3. Dynamic curves of survival (untransformed data)

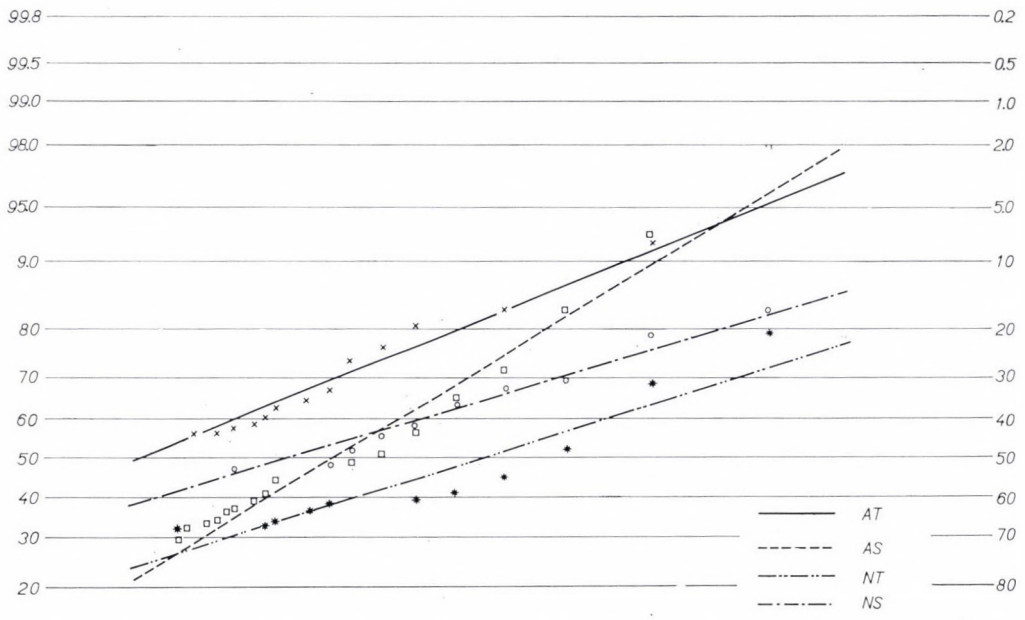

Fig. 4. Relative mortality rate plotted against time. (1/day $\times$ 100 [i.e. the velocity] is plotted on the time-scale. Survival rate is plotted on the frequency-scale)

truth. Nevertheless, they can be used in comparative work. As Fig. 4 indicates, the distributions of the newborn thymus, adult thymus and newborn spleen can be clearly distinguished, and although with all three of them the average survival is around +7 days, and the t test shows no significant differences between them, variance analysis by the F test yields a very highly significant result ($p < 0.1$ per cent). On the basis of the t test, the average survival of $9\frac{1}{2}$ days obtained for the adult spleen is very highly significantly different ($p < 0.1$ per cent) from all three of them.

If we continued the experiments in similar material over 30 days to determine more completely the survival times, and the first few days, too, were taken into account, in analogy to the "natural mortality", the results would be much more reliable. This way, however, it may merely be surmised that as a result of the above corrections the average survival times would be around 12 days in the case of the newborn thymus, 17—18 days in that of the adult and newborn spleen, and over 40 days in the case of the adult thymus. Thus, it is a fact that the adult thymus has a much weaker effect than the newborn thymus, but the adult spleen cannot be called "stronger" than the newborn spleen, its effect is merely delayed, as compared with the other three, and therefore it acts more potently in the later period, by the time the main periods of action of the others are over.

In an earlier paper [11] we have suggested that the thymus, as a phylogenetically and ontogenetically ancient organ, carries with it certain potencies and these lead to a specialisation of this organ in adult age. To this we may add now that apparently it is always the phylo- and ontogenetically most ancient defensive organ that carries with it all the defensive potencies, which it distributes later in connexion with specialisation. This is indicated among others by the fact that for example in birds the extirpation of the bursa of Fabricius produces the same effect as thymectomy in the mammals from the point of view of the immune system [17, 21, 24], while thymectomy has no sequelae at all in birds [25].

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ÜBER DIE IMMUNOLOGISCHE KOMPETENZ VON THYMUS UND MILZ BEI NEUGEBORENEN UND ERWACHSENEN RATTEN

Es wurde an Ratten mit der »runt-disease« Metode die immunologische Kompetenz des Thymus und der Milz von neugeborenen und erwachsenen Tieren untersucht. Dabei konnte festgestellt werden, dass in Übereinstimmung mit den Befunden MILLERs der Thymus von neugeborenen Tieren stark schädigt, während die »runt-disease«-Wirkung des erwachsenen Thymus bedeutend geringer ist. In Gegensatz dazu hat Milzgewebe von erwachsenen Tieren eine starke Wirkung, während die der Milz von Neugeborenen etwa der Wirkung des erwachsenen Thymus entspricht. Daraus wird der Schluss gezogen, dass der Thymus seine immunologischen Potenzen im Laufe der Entwicklung verliert, bezw. den lymphatischen Organen übergibt. Es werden mutmassliche phylo- und ontogenetische Ursachen dieser Erscheinung besprochen.

ИММУНОЛОГИЧЕСКАЯ КОМПЕТЕНЦИЯ ЗОБНОЙ ЖЕЛЕЗЫ И СЕЛЕЗЕНКИ У НОВОРОЖДЕННЫХ И ЗРЕЛЫХ КРЫС

Исследовали иммунологическую компетенцию зобной железы и селезенки у новорожденных и зрелых крыс. В согласии с результатами *Миллера* нашли выраженную компетенцию в зобной железе у новорожденных особей, которая передается селезенке и лимфатическим узлам, когда животные позреливают. Одновременно эти потенции большей частью исчезают из зобной железы. Обсуждаются фило- и онтогенетические причины явления.

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PHENOMENON OF AGEING IN UNIONIDAE, AS EXAMPLE OF AGEING IN ANIMALS OF TELOMETRIC GROWTH

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Synopsis

For ascertaining specific features of types in telometrically growing animals, changes connected with age have been studied in freshwater molluscs (*Anodonta cygnea* and *Unio pictorum*). The variability of the *ovary* increases in old age; the histological picture shows three types: (a) intact ovary filled with oocytes (this is also characteristic of young animals); (b) the ovary is more or less intact, but the cytoplasm of the oocytes shows affinity for methylgreen too and contains dispersed pyroninophilic clumps; (c) disorganized atrophied ovary with egg-cells containing a small number of vacuoles and many yolk granules. The number of follicles and of oocytes per unit of space does not decrease, while the total number of oocytes and the quantity of glochidia in the gill increase in old age. No microscopical involutionary change was observed in the gonads of male specimens. Senile changes in the calcium and pigment contents, further those of the periostractum and the capacity of sedimentation are also described.

Introduction

There are two opposite views regarding the phenomenon of ageing. Some regard it as a universal biological law, others as a pathological process. The first theory demands that phenomena of ageing appear also in telometrically growing animals, i.e. those growing throughout their life. Accordingly, the investigation of senescence in telometrically growing animals is a biologically fundamental problem [13]. Since mussels are telometrically growing animals, it is apparently easy to use them for the study of the problem under discussion. The investigations are, however, made difficult by the fact that it is not easy to determine the age of the animals under natural conditions.

Material and method

Between 1959 and 1963, about 2500 specimens of *Unio pictorum*, *U. tumidus*, *Anodonta cygnea*, *A. complanata* and *A. anatina* were collected from a branch of the Danube by Soroksár. Their age was determined on the evidence of the shells from which the soft parts had been removed. After statistically evaluating the ratio of the distance of the true annual growth rings, we established the characteristic annulus-pattern of the *Unio pictorum*, *Anodonta cygnea* and *Anodonta complanata* under the above mentioned hydrological circumstances. The rate of ovulation was inferred from the histological picture of the ovaries. Two to thirteen year-old specimens of *Anodonta cygnea* were killed in 60° C water; after dividing their adductors, that portion of the foot which contained the gonads was fixed in 5 per-cent formalin, Carnoy's

fluid and Tellyesniczky's potassium bichromate solution. After alcoholic dehydration and embedding in paraffin, 5 μ-sections were made. The following stains were used: haematoxylin-eosin, Giemsa-Pappenheim, Mallory, van Gieson, Foot's impregnation, PAS, TriPAS, methyl-green-pyronin, and the Feulgen reaction. Using a filar ocular micrometer, we determined for each age group the number (per 3 mm^2) of the ripe and ripening oocytes in the lumen, and the oogonia in the wall of the follicles, further the number of follicles in the ovary. The question as to how far the oocytes of old mussles are suitable for fertilization and growth was decided by comparing the gill of young with that of old females. In *Anodonta cygnea*, eggs migrate the intersepta of the gill during August—September so that the number of glochidia developing there can be ascertained from November to the beginning of April. After the opening of the gills, the larvae were washed with warm glycerine, centrifuged for fifteen minutes at 8000 r. p. m., digested with a 33 per cent solution of potassium hydrate, washed with distilled water and then heated. The glochidium shells were weighed to four decimals; following this, we established their number from samples of 2—3 mg and referred it to the whole mass. The result allowed conclusions to the fertility of eggs derived from young and old individuals.

Results

On the evidence of our investigations and the literary data it has been ascertained that the age of Unionidae living in Hungarian waters (of which *Unio pictorum* and *Anodonta cygnea* have been examined in large numbers), does not exceed 13 to 14 years. Exact values cannot be given as the last annuli are closely packed and blurred; their mechanical abrasion can, moreover, falsify the results. Besides, it is possible that, after reaching a certain number of annuli, the mussels live on for years without growing. The size of fresh-water mussels affords no exact information about their age; the oldest examined animals were usually small. Size seems to depend more on locality, nutrition and other hydrological factors and less on age. In the mussels — the age of which was presumably more than 9 to 10 years — a conspicious abrasion of the periostracum, a deposit of lime-granules especially in the branchial lamellae and the papillae of the incurrent orifice and the accumulation of lipochrome pigments in the visceral mass were observed. Since the interconnection of these phenomena cannot be appreciated with accuracy, we concentrated our attention on changes in the phenomena of life and those changes in the gonads which were supposed to be conditioned by age.

It was shown in earlier reports [6, 7] that the degree of sedimentation was proportional to size; we, therefore, compared sedimentation in mussels of identical size but different age. It was found [8] that the sedimentation-rate of older animals was below that of young ones during the first 2 to 3 hours; the difference was particularly striking under unfavourable conditions of oxidation.

The majority of the animals were alternating types: (i) in the majority the follicles were packed with ripe oocytes; the cytological picture corresponded to that of oocytes in young animals (Fig. 1). (ii) In some of the old animals there were hardly any ripe oocytes; we found only smaller cells near the follicles, and they stained mauve with methylgreen-pyronin. The most characteristic

feature of these cells is that their cytoplasm includes numerous scattered clumps; histochemical investigations have shown the latter to contain ribonucleic-acid (Fig. 2). Similar cells appear also in younger animals, but these are homogeneous and less numerous. Since the above-mentioned cells (those near the wall of the follicles) are smaller than the mature ones, and since we saw all transitory forms between these cells and the oogonia, we regard them as immature oocytes. The ovary is strongly disorganized in the third group of

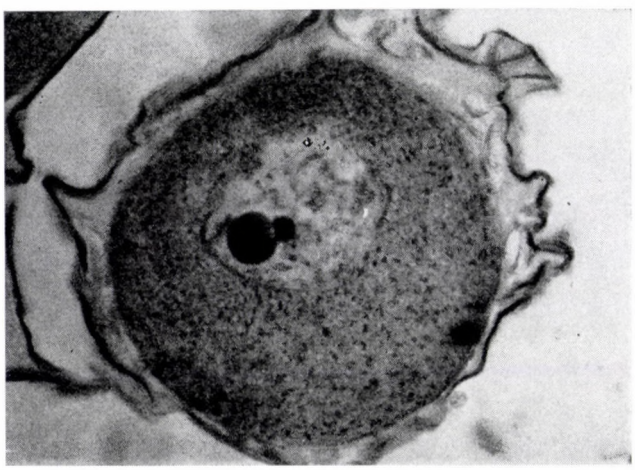

Fig. 1. Mature oocyte from the ovary of a young animal. Also to be found in some old specimens. Haematoxylin-eosin. ×1050

old mussels. Eosinophilic and pyroninophilic granules are accumulated. The ovaries contain hardly any oocytes, the existing ones are deformed, filled with vacuoles, and present the picture of cellular necrosis (Fig. 3). In the given biotope and at the given time, such cells occurred exclusively in old animals. In the oocytes of old mussels, beside the pyroninophilia of the cytoplasm, a light affinity to methylgreen was observed. The cytoplasm was definitely Feulgen-negative, and it is an open question whether this property is due to factors in the metabolism of nucleic acid. It should be noted that, according to RAVEN [9], the nucleus is Feulgen-negative in Lamellibranchiata during the growth of the oocytes.

As the propagation of mussels is seasonal, we compared the August specimens with samples collected at different periods of the year. It was found that ovaries emptied after September were very similar to the above-described third type irrespective of age.

But for a few exceptions, there was no difference between young and old males in respect of spermatogenesis.

Quantitative examination showed that the number of mature and maturing oocytes per unit of space did not decrease with age (Table 1). In old animals, however, the number of oocytes in the follicles was found to vary: some follicles

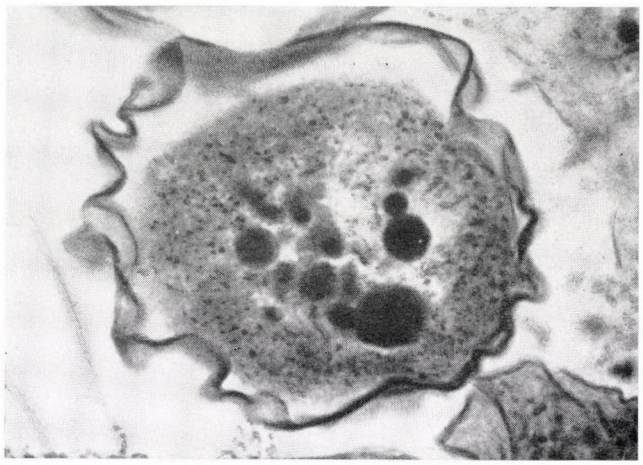

Fig. 2. Oocyte from the ovary of an old animal. Note pyroninophilic clumps in the cytoplasm. Methyl green-pyronin. ×1000

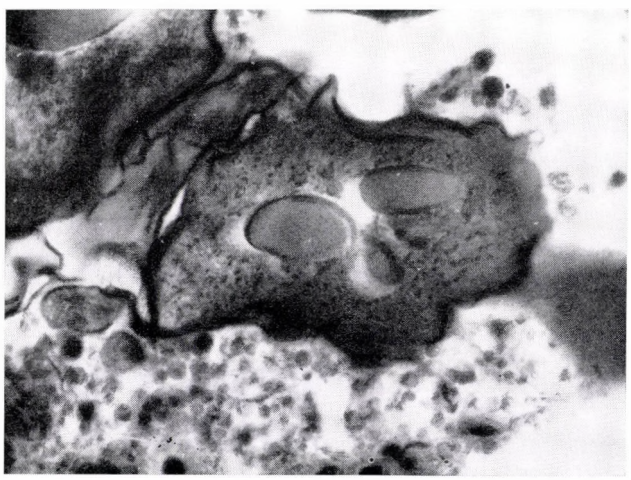

Fig. 3. Oocyte with vacuoles in the atrophized ovary of an old animal. Giemsa-Pappenheim. × 950

contained, on an average, 0.95, others 4.95 egg cells, whereas their number was found fairly uniform in young animals.

In order to study the fertility and development of the oocytes, we made comparative investigations in October and November regarding the quantity

of glochidia in the gills of young and old females (Table 2). The number of glochidia in old animals (referred to size) showed no significant decrease. Thus, in contrast to planarians, the oocytes of old mussels are not infertile and the

Table 1

Quantitative measurements in the ovary of young and old specimens of Anodonta cygnea

Age (years)	Number of follicles/3 mm²	Number of oocytes/3 mm²	Ratio oocyte/follicles
1	31	69	2.23
1	18	80	4.45
1	13	41	3.15
1	10	17	1.75
2	17	21	1.23
2	17	34	2.00
3	22	45	2.04
3	37	121	3.28
3	16	66	4.11
3	21	45	3.24
3	29	51	1.76
3	30	48	1.60
4	19	33	1.74
4	20	39	1.95
4	28	96	3.43
5	28	71	3.09
5	18	42	2.56
6	25	66	2.64
8	23	42	1.83
9	31	34	1.10
10	28	29	1.04
11	19	18	0.95
11	18	42	2.34
11	22	109	4.95
11	15	21	1.40
13	35	50	1.43

process of gastrulation and the development of glochidia are continuous. Whether the viability, e.g. the power to adhere to the organism of the host-fish is equal to that of young animals, is still undecided.

Discussion

Data concerning the life-span of mussels have been compiled by HAAS [5], BRANDER [2] and COMFORT [3]. Their results respecting Anodonta and Unio are in agreement with our findings. They too deem the separation of annuli and pseudoannuli necessary and emphasize the discrepancy between size and age. Investigations in this respect have also been carried out by Hungarian workers [4, 10].

Table 2

Number and weight of glochidia in the gills of variously aged specimens of Anodonta cygnea

Age (years)	Shell			Glochidia	
	length (mm)	width (mm)	height (mm)	weight (g)	number
3	80	25	51	0.3091	410 400
3	95	37	61	0.1944	66 500
3	78	23	51	0.1780	126 000
3	119	36	75	0.3918	80 700
3	112	36	60	0.4745	306 000
4	118	39	74	0.6205	208 000
4	127	42	73	0.4556	148 900
4	110	33	62	0.1545	63 700
4	103	30	54	0.2390	66 000
5	113	42	61	0.4041	221 000
5	86	31	56	0.2418	39 800
5	130	62	80	0.6140	197 600
7	120	42	67	0.1080	93 500
8	154	50	67	1.1946	746 000
9	157	57	81	0.7963	632 000
9	135	45	69	0.3248	187 100
9	146	50	71	0.5725	386 300
10	130	52	74	0.4448	32 800
12	140	52	74	0.8915	469 000

The present morphological and histological investigations have shown that the last phase in the life of mussels differs from that of mammals among others in this that the process of spermatogenesis and the production of viable oocytes continue even in quite old mussels.

A certain degree of involution can be observed in mussels nevertheless. It manifests itself partly by morphological signs and partly physiologically by

a weakening of sedimentation. Its further symptom is the difference in the ripening of the egg-cells and an increased variability of the oocytes.

All this goes to show that phenomena of ageing occur even in telometrically growing mussels which produce oocytes till the end of their life. The biological law of senescence applies to these animals as well, even if its manifestations are not the same as in animals of the higher orders. Further investigations are necessary to ascertain the factors leading to the death of mussels which have completed their potential age of life.

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ALTERUNGSERSCHEINUNGEN BEI FLUSSMUSCHELN (UNIONIDAE) ALS BEISPIEL DER ALTERUNG VON TELOMETRISCH WACHSENDEN TIEREN

Zur Feststellung der Eigenheiten bei den verschiedenen Alterungstypen telometrisch wachsender Tiere wurden die vom Alter bedingten Änderungen der *Anodonta cygnea* und *Unio pictorum* untersucht. Die Änderungen sind die folgenden: bei alten Tieren steigt die Variabilität des Ovariums, das histologische Bild zeigt drei Typen: (a) intakter, mit Oozyten gefüllter Eierstock (dies ist auch für die jungen Tiere charakteristisch), (b) eigentlich intakter Eierstock, doch es besteht eine Affinität des Plasmas der Oozyten auch gegenüber Methylgrün und es sind in ihm dispergierte pyroninophyle Schollen zu sehen, (c) desorganisierter, atrophischer Eierstock, mit einigen (von Vakuolen gefüllten) Eizellen und vermehrten Dotterkörnchen. Die Zahl der auf eine Flächeneinheit entfallenden Follikeln bzw. Oozyten bleibt unvermindert, die Gesamtoozytenzahl und die Menge der in den Kiemen vorhandenen Glochidien nimmt im Alter zu. In den Geschlechtszellen der männlichen Muscheln lassen sich keine Involutionserscheinungen nachweisen. Es werden außerdem die altersbedingten Veränderungen des Kalk- und Pigmentgehaltes, des Periostracums und der Sedimentationsfähigkeit beschrieben.

ЯВЛЕНИЯ СТАРЕНИЯ У UNIONIDAE КАК ПРИМЕР СТАРЕНИЯ ЖИВОТНЫХ С ТЕЛОМЕТРИЧЕСКИМ РОСТОМ

В целях установления типовых свойств старения животных с телометрическим ростом авторы исследовали связанные с возрастом изменения **Anodonta cygnea** и **Unio pictorum**. Эти изменения следующие: вариабельность яичника у старых животных повышается, гистологическая картина показывает 3 типа: (а) неповрежденный, наполненный ооцитами яичник (это характерно также для молодых животных), (б) неповрежденный яичник, но плазма ооцитов имеет сродство также с метиленовой зеленью и в ней обнаруживаются диспергированные пиронинофильные глыбы, (в) дезорганизованный, атрофированный яичник с небольшим количеством ооцитов, наполненных вакуолями и повышенным количеством желточных зерен. У старых животных число фолликулов или ооцитов на единицу площади не снижается, а количество глохидиев в жабрах и общее число ооцитов повышается. В половых железах мужских раковин под микроскопом инволюционных изменений выявить не удалось. Кроме этого авторы описывают изменения содержания извести и пигмента, периостракума и седиментационной способности, наблюдаемые у старых животных.

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EFFECT OF COLCHICINE ON THE CARBOHYDRATE METABOLISM DURING FORMATION OF MYCELIAL FELTS OF CUNNINGHAMELLA SP.

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Synopsis

Incubation of *Cunninghamella* spores in Richard's solution containing 5, 10 and 20 p.p.m. colchicine for 5 days at 25° C did not affect the dry weight of the produced mats. Furthermore, the highest concentration of colchicine favoured sucrose absorption while the other treatments had no effect. In all cases, glucose was absorbed in surplus.

Analysis of the fungal mats proved that low concentrations of colchicine favoured polysaccharide synthesis particularly the glucosans and galactosans while the soluble sugars seemed to be unaffected. On the other hand, 20 p.p.m. colchicine favoured the accumulation of the absorbed sugars in the form of disaccharides and lowered the conjugated pentoses content. Furthermore, the comparatively small increase of the glucosans and galactosans was accompanied by a remarkable decrease in the pentosans; an indication of the inhibitory effect of the drug on nucleotide formation.

Introduction

Data is accumulating on the effects of colchicine on the living organisms. All reports are in favour of the fact that colchicine induced a lowering in the rate of respiration [2, 14] accompanied by an increase in most of the carbohydrate and nitrogen fractions of the higher plants e.g. cabbage, tomato, barley and sweet potato [1, 4, 7]. In animals colchicine caused the inhibiton of the dehydrogenases and esterases accompanied by a loss in dry weight [3, 6, 8, 10, 16].

Very little has been published on the effects of colchicine on fungi. In a previous publication, the author [12] found that colchicine caused a persistent inhibitory effect on CO_2 output by the fungal hyphae even after the transfer of the mats to fresh media without colchicine. Accordingly, it was thought to study the effect of this drug on the carbohydrate metabolism of the fungus under test.

Material and methods

The fungus used in this experiment, *Cunninghamella* sp. was isolated from the Egyptian garden soil, purified and kindly supplied by Dr. A. M. SALAMA. Stock cultures were maintained at 25° C on Dox slopes. The fungus was subcultured every two weeks for the inoculation purposes.

In experimental work, the fungus was grown in 250 ml. conical flasks containing 50 ml of modified Richard's solution of the following composition:

Sucrose	20 mgs
Pot. dihydrogen phosphate	5 gms
Ferrous sulphate crystals	0.1 gm
Potassium nitrate	10 mgs
Magnesium sulphate crystals ..	2.5 gms
Distilled water to	1000 ml.

After sterilization and cooling of the culture medium, colchicine in 5, 10 and 20 p.p.m., was added aseptically and cautiously. The flasks were then inoculated with 0.2 ml concentrated suspension from 7 days old fungal slants and incubated at 25° C for 5 days. The produced mats were dried at 70° C till constant dry weight and both the mats and media were analyzed for their carbohydrate content according to the methods previously mentioned by the author [11, 13]. At least 5 replicates were used in each treatment.

Experimental results

Effect of colchicine on dry weight

The average values of the dry weight of the colchicine-treated mats are recorded in Table 1. The results indicate that colchicine, up to 20 p.p.m., had very little, if at all, effect on the mycelial dry weight of *Cunninghamella* grown for 5 days.

Effect of colchicine on sucrose uptake

The uptake of glucose and fructose components of sucrose, as well as the total sugar uptake by *Cunninghamella*, during its growth, is represented in Table 2. The table shows that 5 and 10 p.p.m. colchicine seemed to have no effect on sugar uptake while 20 p.p.m. enhanced this process. In all cases (control or treated samples) the growing fungus absorbed more of the glucose than the fructose component of sucrose, a phenomenon that was furthered in the presence of the high concentration of colchicine.

Table 1

Dry weight of mycelial mats of Cunninghamella sp. *spores suspended in media containing different concentrations of colchicine*

(Expressed in mgm)

Medium	Dry weight
Richard's solution	277±20.6
Richard's + 5 p.p.m. Colchicine	280±18.0
Richard's + 10 p.p.m. Colchicine	241±23.3
Richard's + 20 p.p.m. Colchicine	243±19.2

Table 2

Total sucrose uptake as well as uptake from the glucose and fructose components of sucrose by Cunninghamella sp. *grown on media containing different concentrations of colchicine*

(Expressed as mgm per mat)

Medium	Sucrose absorption		
	Glucose	Fructose	Total
Richard's solution	64.0	29.3	93.3
Richard's + 5 p.p.m. Colchicine	60.6	28.9	89.5
Richard's + 10 p.p.m. Colchicine	61.8	27.2	89.0
Richard's + 20 p.p.m. Colchicine	84.1	27.2	111.3

Carbohydrate analysis of the tissues

The mean values of the results of analysis of the differently treated samples are recorded in Table 3. It shows that colchicine did not cause any appreciable change in the total soluble sugars except when present in 20 p.p.m. concentration where the mats accumulated large amounts of the dissacharides while the monosaccharide content was very much lowered.

The initial hexose content of the fungal hyphae was nearly one third the total monosaccharides and was kept as such in the presence of the low concentrations of colchicine. On the other hand, 20 p.p.m. increased this ratio to half the monosaccharide content. In all cases, the fructose component of the monosaccharides was nearly double the glucose; a ratio that seemed to be unaffected by the presence of colchicine. Further, the high concentration of colchicine seemed to reduce the pentose content of the tissues to nearly one third the initial; the decrease being mostly from the conjugated fraction; an indication that this concentration of the drug had lowered the mononucleotide content of the fungal hyphae.

Table 3

Average values of the results of analysis of mycelial mats of Cunninghamella sp. *suspended in different concentrations of colchicine*

(expressed as mg sugar per mat)

Medium	Glucose	Fructose	Total hexose	Free pentose	Conjugated pentoses	Total pentoses	Total mono-saccharides
Richard's solution	1.31	2.73	4.04	3.02	4.50	7.52	11.56
Richard's + 5 p. p. m. colchicine ...	1.23	2.49	3.72	2.93	4.37	7.30	11.02
Richard's + 10 p. p. m. colchicine ...	1.20	2.36	3.56	2.87	4.41	7.28	10.84
Richard's + 20 p. p. m. colchicine ...	0.82	1.81	2.63	2.04	0.74	2.78	5.42

Medium	Disaccharides	Total soluble sugars	Glucosans	Fructosans	Galactosans	Total hexosans	Pentosans	Total polysaccharides	Total carbohydrates
Richard's solution	16.20	27.76	1.95	3.01	3.67	8.63	6.44	15.07	42.83
Richard's + 5 p.p.m. colchicine	16.42	27.44	5.53	3.82	7.55	16.90	6.00	22.90	50.34
Richard's + 10 p.p.m. colchicine.....	17.28	28.12	5.51	3.14	7.53	16.18	6.28	22.46	50.58
Richard's + 20 p.p.m. colchicine	41.12	46.53	5.53	3.14	7.96	16.63	3.55	20.18	66.71

Invertase hydrolysis of the fungal extracts did not show the presence of sucrose. On the other hand, acid hydrolysis, in 1 *N* total acid concentration at 100° C for 1 hour, proved the presence of large amounts of other disaccharides; a value that remained nearly unaffected in presence of the lower concentration of colchicine. On the contrary, 20 p.p.m. of the drug seemed to induce the excessive accumulation of this fraction inside the tissues.

The total polysaccharide content of the control samples formed about one third the total carbohydrates. This ratio increased in presence of the lower concentrations of colchicine but seemed unaffected in presence of 20 p.p.m. of the drug.

Furthermore, the presence of colchicine (in any concentration tested) caused the doubling of the total hexosans of the fungal cells; the increase being mostly in the glucosans and galactosans while the fructosans seemed to be unaffected. Furthermore, the galactosans were nearly double while the glucosans were trebled, irrespective of the colchicine concentration.

On the other hand, the pentosan content of the fungal hyphae seemed to be unaffected by the presence of the lower concentrations of colchicine. However, 20 p.p.m. induced a rapid decrease of this fraction to half the initial value. In all cases colchicine lowered the ratio of pentosans to hexosans particularly in presence of 20. p.p.m. concentration.

Discussion

The results of this experiment show that colchicine had no effect on the dry weight of the fungal mats during their growth. Further, sucrose uptake was unaffected by the presence of the lower concentrations of the drug but the higher doses seemed to be stimulatory to this process, an indication that, under the latter conditions, colchicine is stimulatory to phosphorylases. In all cases, absorption of glucose was in surplus over fructose; a phenomenon already noticed by TOLBA and SALAMA [18, 19] using *Rhizoctonia solani*.

Tissue analysis proved that, inspite of the fact that the total carbohydrate content of the fungal mats grown on Richard's media containing 5 or 10 p.p.m. colchicine was more or less identical with that of the control samples, yet both concentrations of the drug favoured the accumulation of polysaccharides particularly the glucosans and galactosans; a phenomenon that suggests the activation of phosphorylase and hexose isomerase. On the other hand, the excessive sugar absorption recorded in presence of 20 p.p.m. colchicine was accompanied by a remarkable increase in the disaccharide content. This was followed by a lowering of the monosaccharide level particularly the conjugated pentoses; an observation that leads to the suggestion that this treatment though enhanced the phosphorylase activity yet retarded the formation of nucleotides. This was further observed in the polysaccharide fraction where the pentosans were reduced to half the normal while the glucosans and galactosans were identical to those recorded for the other treatments; an indication that 20 p.p.m. further inhibited the nucleoprotein formation.

It is worth noting that the ratio of polysaccharide to total carbohydrates increased, in presence of low concentrations of colchicine, but was more or less unaffected in presence of the higher concentration. This proves that low concentrations of the drug favoured polysaccharide building inspite of normal sugar absorption while the higher concentration did not affect the process. The increased polysaccharide content of such treated samples was merely proportional to the increased sugar absorption while most of the sugars absorbed accumulated as disaccharides.

In this connection it may be mentioned that SULLIVAN [17], EKDAHL [4] and BARR [1] showed that sucrose content was higher in tetraploid than diploid plants. LETTRÉ [9] proved that colchicine destroyed the adenosine triphosphate of the cells while SKIPPER [15] claimed that colchicine is ineffective as an inhibitor for nucleic acid synthesis in rats. On the other hand, FRIEDMAN [5] proved that colchicine increased the deoxyribonucleic acid and decreased the ribonucleic acid of the tumour cells. Furthermore, KOGA [7] proved that colchicine favoured starch synthesis in sweet potato.

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DIE WIRKUNG DES COLCHICINS AUF DEN KOHLENHYDRATSTOFFWECHSEL WÄHREND DER BILDUNG DES MYCELIUMS BEI CUNNINGHAMELLA SP.

Die Inkubation von *Cunninghamella* Sporen mit 5, 10 und 20 ppm Colchicin enthaltender Richards Lösung hatte auf das Trockengewicht des erzeugten Myceliums keinen Einfluss. Weiterhin hatte die grösste Colchicinkonzentration die Rohrzuckerabsorption bevorzugt, während die anderen Behandlungen keine Wirkung ausübten. In allen Fällen wurde Glucose in Überfluss absorbiert. Analysen der Pilzmycele bewiesen, dass niedrige Colchicinkonzentrationen die Polysaccharidsynthese, speziell die der Glucosanen und Galaktosanen fördern, während die unlöslichen Zucker nicht beeinflusst werden. Andererseits förderte 20 ppm Colchicin die Anhäufung von absorbierten Zuckern in Form von Disacchariden und verminderte den polymerisierten Pentosegehalt. Weiterhin wurde die verhältnismässig geringe Zunahme der Glukosanen und Galaktosanen von einer beachtenswerten Abnahme der Pentosanen begleitet; das spricht für die hemmende Wirkung der Verbindung auf die Nucleotidsynthese.

ВЛИЯНИЕ КОЛХИЦИНА НА УГЛЕВОДНЫЙ ОБМЕН В ТЕЧЕНИЕ ОБРАЗОВАНИЯ МАССЫ МИЦЕЛИЯ У CUNNINGHAMELLA SP.

Споры *Cunninghamella sp.* инкубировали на среде Рихарда, содержащей 5; 10 и 20 колхицина, в течение 5 дней при 25°. На сухой вес образовавшейся массы мицелия обработка колхицином не влияла. Самая высокая концентрация кодхицина способствовала поглощению сахарозы, в то время как остальные концентрации оказались неэффективными. Глюкоза была поглощена в каждом случае в избытке. Анализ массы мицелия показал, что малые концентрации колхицина благоприятно влияют на синтез полисахаридов, особенно глюкозанов и галактозанов, в то время как на растворимые сахара не оказывают влияния. С другой стороны колхицин в концентрации 20 ppm. благоприятно влияет на накопление поглощенных сахаров в дисахаридной форме и снижает содержание полимеризированной пентозы. Кроме этого, параллельно сравнительно небольшому увеличению содержания глюкозанов и галактозанов, в значительной степени снизилось количество пентоз; этот факт указывает на то, что колхицин оказывает тормозящее действие на образование нуклеотидов.

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EFFECT OF KINETIN ON ENZYMES OF GLYCOLIC ACID METABOLISM IN CEREAL LEAVES

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Synopsis

The activity of glycolic acid oxidase and glyoxylic acid reductase has been measured in homogenates from detached barley and wheat leaves. It was found that the root system exerts an indirect regulatory effect on the level of glycolic acid oxidase and glyoxylic acid reductase in the leaf tissues. The activity of glycolic acid oxidase was markedly reduced in detached leaves. By contrast, the activity of glycolic acid reductase increased upon detachment. Treatment of detached leaves with 10^{-5} M kinetin restored the activity of glycolic acid oxidase to the level found in intact control leaves. Glyoxylic acid reductase was not markedly affected by kinetin-treatment. Considerable amounts of glycolic acid oxidase proenzyme were found in the leaf tissues. The proenzyme could be activated by adding 10^{-4} M FMN to the homogenates. In contrast to the holoenzyme, the amount of proenzyme was not reduced in detached leaves. Kinetin had no major effect on proenzyme level.

Introduction

Kinetin (6-furfurylaminopurine) affects a number of physiological processes in higher plants including cell division, various aspects of development, transport phenomena, heat tolerance and respiration [1, 3, 6, 16, 19, 21, 23, 35]. However, the interest centers around the effect of kinetin on protein and nucleic-acid metabolism [14, 15, 24, 27, 30, 35, 36] as it is suspected that nucleic-acid and protein metabolism might be the primary point of attack of kinetin. It is surprising, therefore, that little attention has been devoted to the effect of kinetin on enzymes, except some work on the influence of kinetin on nucleases [17] and xanthine oxidase [5] *in vitro*. In the present study, the *in-vivo* effect of kinetin on the level (activity) in detached leaves of two distinct proteins, glycolic acid oxidase and glyoxylic acid reductase, has been tested.

It was demonstrated in a previous work that the root system exerts a regulatory effect on the enzyme level in leaf tissues. Evidence has been presented that the activity of a number of enzymes increases in the leaf tissues upon removal of the root system [4]. An exception to this rule is starch phosphorylase and glycolic acid oxidase, the activity of which decreases with the breakdown of protein in the yellowing leaves [4]. As the level of proteins in detached leaves can easily be regulated with kinetin, and a net protein and chlorophyll synthesis can be induced in kinetin-treated isolated leaf tissues [24], experi-

ments were started to find out whether or not the glycolic acid oxidase level was correlated with the protein content and chloroplast activity in green leaves. The possible connection of glycolic acid oxidase level with the functioning of chloroplasts is suggested by literary data indicating (*a*) the general absence of glycolic oxidase in roots and tubers [22, 32] *(cf.*, however, the low activity found in rice roots by MITSUI [20] and confirmed in our experience) (*b*) the occasional appearance of glycolic acid oxidase in greening tubers and roots [22, 31], (*c*) the low activity of glycolic acid oxidase in etiolated leaves [2, 8, 9] which greatly increases upon illumination [12, 13, 31].

In addition to the fully active holoenzyme, considerable amounts of glycolic acid oxidase proenzyme are present in most tissues. As shown by KUCZMAK and TOLBERT [13], the addition of flavine mononucleotide (FMN) to cell-free homogenates results in the activation of the inactive proenzyme. The effects of kinetin on the proenzyme level and the enzyme/proenzyme ratio were also determined.

Material and methods

Barley seedlings (Hungarian variety MFB) were grown under ordinary greenhouse conditions; the primary leaves were detached in the two-leaf stage and placed with their bases into tap water and 10^{-5} M kinetin respectively. After 4 to 7 days the leaf tissues were homogenized in a cold mortar in a tenfold amount of 0.1 *M* phosphate buffer, pH 7.8. Leaves of intact plants of the same age served as controls.

Glycolic acid oxidase activity was determined colorimetrically according to KOLESNIKOV [11] on the basis of the amount of glyoxylic acid formed by the homogenates upon the addition of glycolate. In strongly acidic medium, in the presence of phenylhydrazine and $K_3Fe(CN)_6$, glyoxylic acid gives a red colour.

Alternatively, glycolic acid oxidase was assayed by the standard manometric procedure at 30° C in a Warburg constant-volume respirometer on the basis of O_2-uptake obtained upon addition of glycolate to the homogenates [13].

Proenzyme levels were determined according to KUCZMAK and TOLBERT [13]. The difference in O_2-uptake obtained in the manometric assay without and with 10^{-4} M FMN was taken as a measure of the amount of proenzyme present in the extracts.

Glyoxylic acid reductase was assayed spectrophotometrically in cell-free homogenates [38] on the basis of the decrease in absorbancy at 340 mμ in the presence of NADH (nicotinamide adenine dinucleotide, reduced form) and glyoxylate. A Zeiss VSU 1 spectrophotometer was used throughout the experiments.

Average values calculated from a high number of experiments are given in the figures for glycolic acid oxidase activity and proenzyme levels. A representative example is given for the spectrophotometric determination of glyoxylic acid reductase.

Glyoxylic acid and NADH were purchased from Sigma Chemical Co., St. Louis, Missouri. Glycolic acid was obtained from the firm REANAL, Budapest.

Results

The determination of glycolic acid oxidase activity in detached barley leaves indicated a marked decrease in holoenzyme level upon the removal of the root system. The enzyme activity was reduced to about 50 per cent of the controls (leaves of intact plants). Detached leaves kept in 10^{-5} M kinetin

retained their normal glycolic acid oxidase activity (Fig. 1). Both the effect of detachment and that of the kinetin-treatment proved reproducible. Both methods yielded identical results. Experiments were carried out throughout the year with essentially the same results.

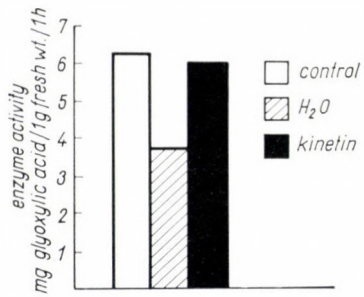

Fig. 1. Effect of detachment and treatment with 10^{-5} M kinetin on the activity of glycolic acid oxidase in barley leaves

Proenzyme levels were also determined by both methods. The colorimetric procedure, for unknown reasons, gave erratic results in the presence of FMN. Therefore, the detailed studies were carried out with the manometric assay.

As may be seen in Fig. 2, the proenzyme level was hardly changed upon detachment. There was a slight tendency for an increase in proenzyme level in the isolated leaves. The proenzyme level was markedly higher than the holoenzyme concentration both in control and detached leaves. Upon detachment, the proenzyme/holoenzyme ratio shifted strongly towards proenzyme concentration. The effect of kinetin on proenzyme level was slight. If any, it was a small reduction of proenzyme concentration in some experiments.

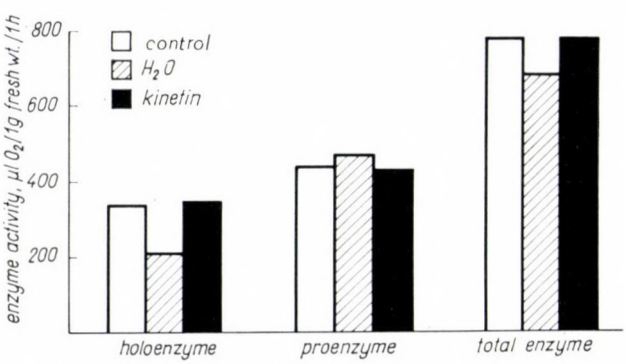

Fig. 2. Effect of detachment and treatment with 10^{-5} M kinetin on the level of glycolic acid oxidase holoenzyme and proenzyme in barley leaves

The effect of detachment and kinetin on the total (pro + holo) enzyme level is also shown in Fig. 2. As the changes in proenzyme concentration were small, the effects of detachment and kinetin on the total enzyme level were similar to those exerted on holoenzyme concentration. The percentage differences in total enzyme concentration due to detachment or kinetin-treatment are, of course, smaller than those encountered in the holoenzyme level.

Results essentially similar to those reported above were obtained in experiments with wheat plants.

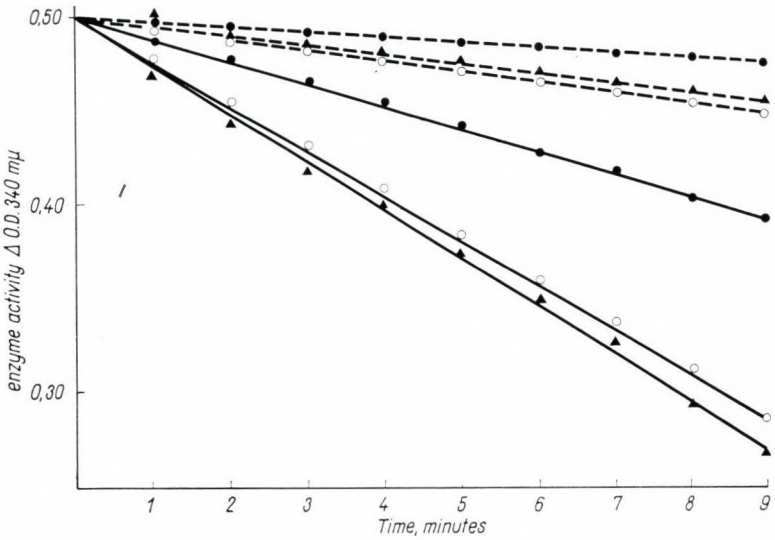

Fig. 3. Effect of detachment and treatment with 10^{-5} M kinetin on the activity of glyoxylic acid reductase in barley leaves. Composition of the system: 0.5 μmole NADH, 20 μmole glyoxylate, enzyme corresponding to 25 mg fresh weight of leaf tissue, 0.1 *M* Tris buffer pH 7.0. Final volume 2.0 ml. Light path 0.5 cm. Composition of the blank: the complete experimental mixture minus glyoxylate. Blank — — —. Experimental ———. ● = Extract from control leaves, ○ = Extract from detached leaves, ▲ = Extract from kinetin-treated leaves

The response to detachment and kinetin treatment of the other key enzyme of glycolic acid metabolism, glyoxylic acid reductase, was entirely different from that given by glycolic oxidase. The activity of glyoxylic acid reductase was markedly increased upon detachment (Fig. 3) similarly to many other enzymes of oxidative metabolism [4]. Kinetin affected the activity of glyoxylic acid reductase to a small extent if at all. It may also be seen in Fig. 3 that the extracts from detached leaves oxidized NADH even in the absence of glyoxylate faster than those prepared from the controls. Although this "NADH-oxidase" activity was weak, the effect of detachment on NADH oxidation was highly reproducible. The difference in the speed in NADH oxidation in the presence and in the absence of glyoxylate is regarded as a measure of glyoxylic acid reductase activity.

Discussion

The role of the various particulate elements of the plant cell in the synthesis of enzymes and/or in the regulation of their activity is a basic problem of plant physiology. The chloroplasts are known to be important sites of synthetic activities including protein synthesis. The direct role of chloroplasts in the synthesis of enzymes localized in the chloroplasts is obvious. However, the chloroplasts have certainly an indirect effect on the synthesis of cytoplasmic enzymes as well. The localization of glycolic acid oxidase in the plant cell is an open problem. However, previous results and the data presented in this paper strongly favour the view that directly or indirectly the chloroplasts are involved in the regulation of glycolic acid oxidase level and/or activity in the plant cell. Part of the evidence (distribution of glycolic oxidase and the effect of light on glycolic oxidase activity) has been listed in the introduction. It should be added that plant diseases which lead to the yellowing of leaves are associated with a decreased glycolic acid-oxidase activity [7, 28, 29]. The present results also indicate a striking parallelism between yellowing (chloroplast and chlorophyll breakdown) and decrease in glycolic acid oxidase activity in detached leaves. The activity is fully maintained if the detached leaves are treated with kinetin. In the kinetin-treated leaves the chlorophyll content remains unaltered (or surpasses the control), and the activity of the photosynthetic apparatus is also maintained. The evidence, therefore, strongly supports the idea that the chloroplast apparatus has a fairly direct role in the regulation of glycolic acid oxidase level. As the activity of glycolic acid oxidase depends on the amount of available FMN as well [13, 28], the regulatory role of chloroplasts might consist in (*a*) a stimulation of enzyme protein (proenzyme) synthesis, (*b*) in the stimulation of FMN synthesis, and (*c*) in the regulation of the coupling of FMN to the protein moiety of the enzyme. Indeed, the decrease of glycolic acid oxidase activity in yellowing tissues is evoked by different mechanisms. For example, in some diseased tissues the decrease in active FMN level seems to be alone responsible for the effect. Proenzyme simultaneously accumulates [28]. The light-induced stimulation of glycolic acid oxidase activity is based on both proenzyme and total enzyme synthesis. The total level of flavins is also increased upon illumination [13]. As shown by the present study, in detached yellowing leaves the decrease in enzyme activity is primarily due to a decrease in holoenzyme level. The small accumulation of proenzyme found in some experiments might be due to a release of proenzyme from the holoenzyme. Under the effect of kinetin, the holoenzyme level remains constant after detachment. As kinetin stimulates protein synthesis, this observation might reflect a kinetin-induced synthesis of holoenzyme.

It is interesting that, in contrast to glycolic oxidase, the activity of glyoxylic acid reductase increases in detached leaves. The shift in the activity

of these two enzymes might induce a profound effect on the level of glycolic acid in plant tissues. There is some evidence that this is indeed the case [28]. As glycolic and glyoxylic acids are key metabolites for a number of synthetic processes [10, 26, 32], and the glycolic oxidase — glyoxylic reductase system might participate in the terminal hydrogen transport in green leaves [37, 38], the effect of kinetin on glycolic acid oxidase has certainly side-effects in a number of phases of plant metabolism.

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DIE WIRKUNG DES KINETINS AUF ENZYME DES GLYKOLSÄURE-STOFFWECHSELS IN GERSTEN- UND WEIZENBLÄTTERN

Die Aktivität der Glykolsäureoxydase und Glyoxylsäurereduktase wurde in abgetrennten Gersten- und Weizenblättern bestimmt. Es wurde gefunden, dass das Wurzelsystem über die Aktivität der obigen zwei Enzyme in den Blättern eine indirekte, regulierende Wirkung ausübt. Die Aktivität der Glykolsäureoxydase war in den abgetrennten Blättern stark reduziert, und die der Glyoxylsäurereduktase erhöht. Die Behandlung der abgetrennten Blätter mit 10^{-5} M Kinetin hat die Aktivität der Glykolsäureoxydase auf dem Niveau der intakten Kontrollblätter gehalten. Die Glyoxylsäurereduktase-Aktivität erwies sich im wesentlichen als kinetinunabhängig. In den Blattgeweben wurden beträchtliche Mengen von Glykolsäureoxydase-Proenzym gefunden. Das Proenzym konnte durch 10^{-4} M FMN in den Homogenaten aktiviert werden. Im Gegensatz zum Holoenzym war die Menge an Proenzym in den abgetrennten Blättern nicht reduziert. Kinetin hat auf den Proenzymgehalt keinen wesentlichen Einfluss ausgeübt.

ДЕЙСТВИЕ КИНЕТИНА НА ФЕРМЕНТЫ ОБМЕНА ГЛИКОЛЕВОЙ КИСЛОТЫ В ЛИСТЬЯХ ЗЛАКОВЫХ

Авторы определили активность оксидазы гликолевой кислоты и редуктазы глиоксалевой кислоты в гомогенатах изолированных листьев ячменя и пшеницы. Обнаружили, что корневая система оказывает косвенное регулирующее действие на активность оксидазы гликолевой кислоты и редуктазы глиоксалевой кислоты в тканях листа. В изолированных листьях активность оксидазы гликолевой кислоты значительно снижается. Напротив этому, активность редуктазы глиоксалевой кислоты значительно повышается под влиянием изолирования. Кинетин в концентрации 10^{-5} М поддерживает на нормальном уровне активность оксидазы гликолевой кислоты в листьях даже в отсутствии корневой системы. На активность редуктазы глиоксалевой кислоты обработка кинетином не влияет существенно. В тканях листьев обнаружено значительное количество профермента оксидазы гликолевой кислоты. В гомогенатах профермент можно было активировать добавлением флавон-мононуклеотида в концентрации 10^{-4} М. Напротив голоферменту, количество профермента не снижалось в изолированных листьях. Кинетин только в незначительной мере влияет на уровень профермента.

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NEW METHOD FOR ECOLOGICAL COMPARISON OF VEGETATIONAL UNITS AND OF HABITATS

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(Received May 25, 1964)

Synopsis

The method is based on the old concept of plant species as indicators of habitat. Application of numerical scales of ecological values (T, W, R) and calculation of Gruppenanteil or Gruppenmenge coefficients lead to abstract numerical values instead of species or groups of species. Thus global comparison of vegetational units and of habitats and use of mathematicalstatistical methods become possible.

The new method is based on the old indicator concept of the plant species (indicators of habitat). The ecological structure and habitat of plant communities may be characterized with the aid of a 10-degree T (temperature ecology number) scale (new, 1—10, arctic — aequatorial), a 10-degree W (water ecology number) scale refined after POGREBNIAK [2] and a 5-degree R (soil reaction number) scale after ELLENBERG [1]. Ecological structure is to be understood as the proportion of indicator, respectively of ecological species groups partaking in the plant association according to GA or GM ("Gruppenanteil-Gruppenmenge" TÜXEN—ELLENBERG [3]). All the species as well as all their occurence in the phytocenological table are thus taken into account. Employing characteristic-curve traced, a b s t r a c t e d of species, we may effect a g l o b a l comparison of habitats. Mathematical-statistical methods may also be employed (carried out by I. PRÉCSÉNYI). The complete tabular material of five plant communities of a succession series has been investigated in this way as an experiment (from Seslerietum to Querco-Carpinetum, from rendzina to brown soils). The method has also been demonstrated with a single species *(Waldsteinia geoides)*. In can be aptly shown on a multidimensional ecostructure diagram. Our method mediates between the "tabular plant cenology" of Central Europe and the English—American "plant ecology". We think to add by this thought to the preparatory work of the International Biological Program (Sect. A. 1.).

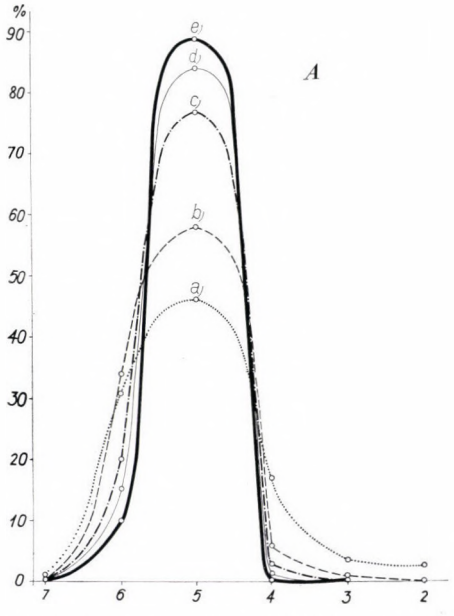


Fig. 1. Characteristic curves of the T *(A)*, W and R *(B)* degrees-values of the five plant communities. The black stripe marks the 95% confidence interval of means of W and R referring to the habitat

Explication and data (calculated by I. PRÉCSÉNYI).

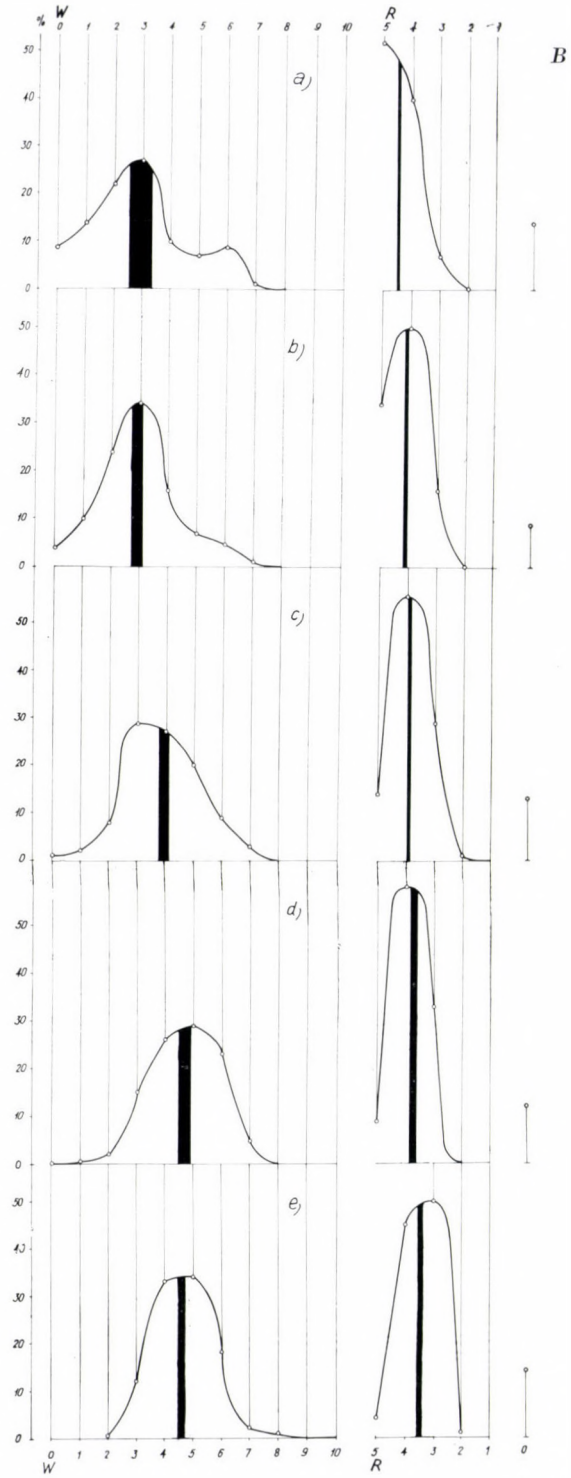
	Sum of GA
a) = Seslerietum	358
b) = Waldsteinio-Spiraeetum	586
c) = Tilio-Fraxinetum	644
d) = Mercuriali-Tilietum	951
e) = Querco-Carpinetum	1269
f) = *Waldsteinia geoides* (ecospectrum by Fig. 3.)	3932

Communities and habitat	*a)*	*b)*	*c)*	*d)*	*e)*
T $\bar{x}$	4.99	5.27	5.16	5.15	5.09
W $\bar{x}$	2.84	2.89	3.93	4.63	4.66
R $\bar{x}$	4.45	4.16	3.85	3.77	3.51
Communities					
W s^2	3.00	1.89	1.66	1.51	1.01
R s^2	0.41	0.47	0.41	0.36	0.35
Habitat*					
W s^2	0.44	0.15	0.07	0.11	0.04
95%	±0.36	±0.20	±0.18	±0.17	±0.08
R s^2	0.03	0.02	0.01	0.01	0.01
95%	±0.09	±0.08	±0.07	±0.04	±0.04

Comparison of communities (by χ^2 test)

	T	W	R
a) — *b)*	$P < 0.1\%$	$P < 0.1\%$	$P < 0.1\%$
b) — *c)*	$P < 0.1\%$	$P < 0.1\%$	$P < 0.1\%$
c) — *d)*	$P < 0.1\%$	$P < 0.1\%$	$P < 5.0\%$
d) — *e)*	$P < 1.0\%$	$P < 0.1\%$	$P < 0.1\%$

* 95% confidence interval refers to habitat means.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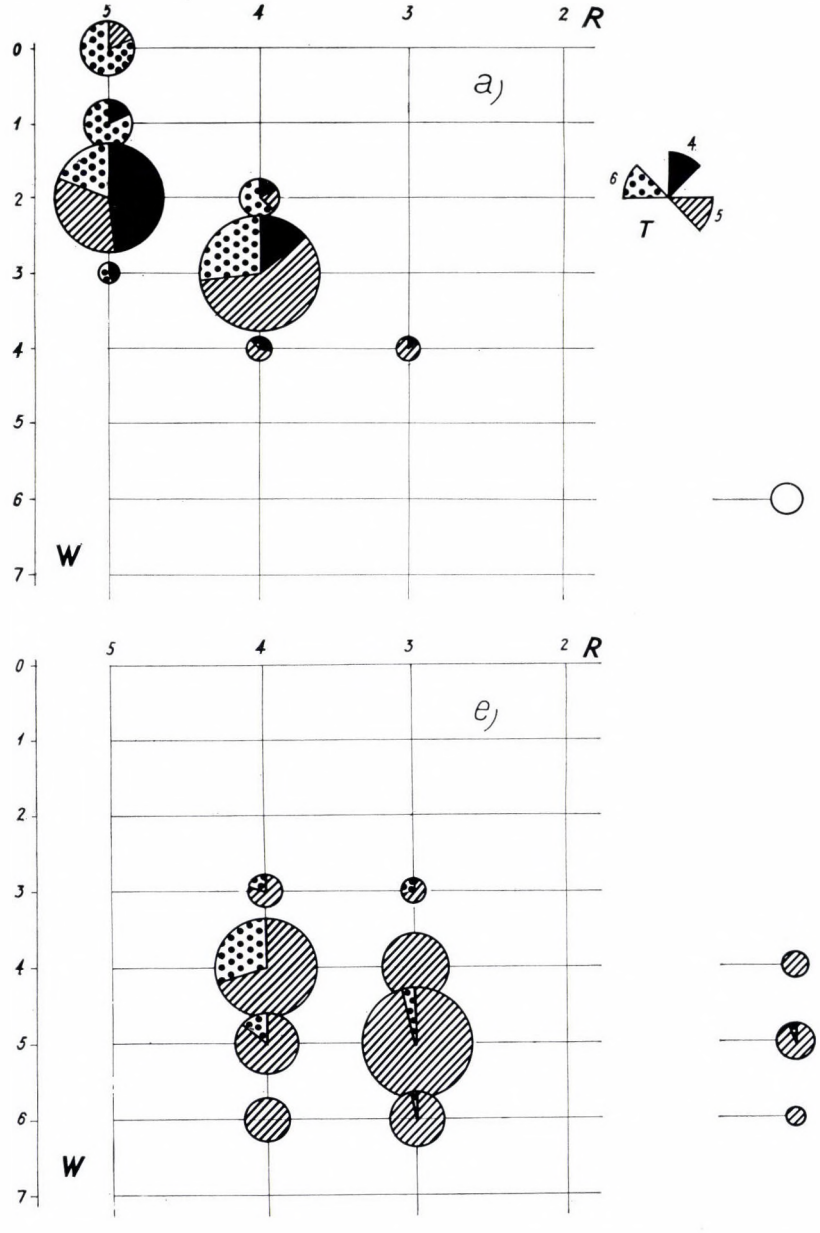
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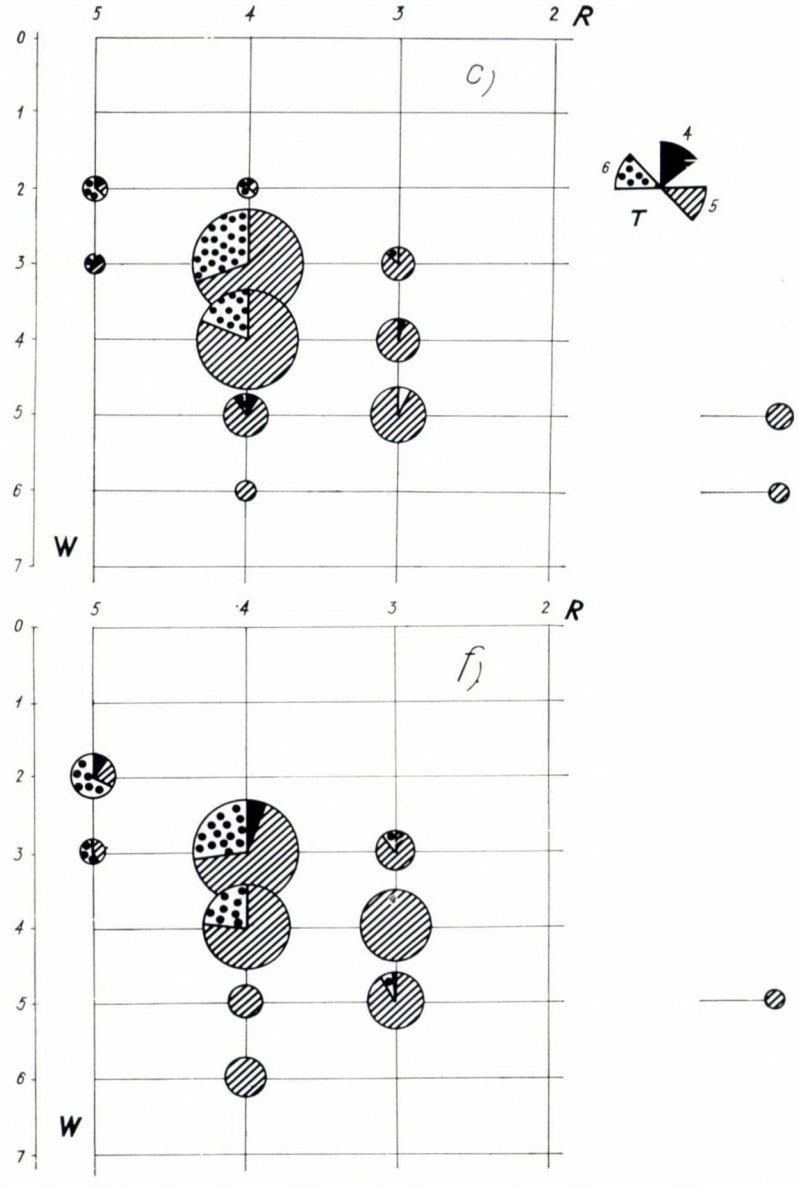

Figs. 2 and *3*. Examples of multidimensional ecostructure diagrams. The % of frequency of the coupled W—R degree-values are equal to the radius of the circle and the T values belonging to them are measured on the circumference of the circle in % of frequency. Percentages under 3 are not indicated. See the explication by the Fig. 1.

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NEUE METHODE ZUM ÖKOLOGISCHEN VERGLEICH DER VEGETATIONSEINHEITEN UND DER STANDORTE

Die Methode geht von der alten Konzeption der Indikator-Arten aus. Durch Anwendung von ökologischen Wert-Skalen (T, W, R) und durch Gruppenanteil (GA oder eventuell auch GM) Berechnung kommt man statt Arten oder Artengruppen zu abstrahierten Zahlenwerten. Dadurch wird ein globaler Vergleich der Vegetatisuseinheiten und der Standorte und die Anwendung der allgemein üblichen Methoden der Mathematischen-Statistik möglich.

НОВЫЙ МЕТОД ДЛЯ ЭКОЛОГИЧЕСКОГО СРАВНЕНИЯ ЕДИНИЦ ВЕГЕТАЦИИ И МЕСТОНАХОЖДЕНИЙ

Метод основан на старой концепции о видах-индикаторах. Используя шкалы экологических величин (T, W, R) и проводя вычксление участие пазных величин в группах (Gruppenanteil или Gruppenmenge) получаются абстрактные численные величины вместо видов или групп видов. Таким образом всесторонное сравиение единип вегетации и местонахождений и примение обычные методы математической статистики становятся возможными.

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RECENSIONES

CLEMENTS, A. N.: **The Physiology of Mosquitoes**
Pergamon Press, Oxford (1963), pp. 393, 80 s.

The results of research and experimental work done on the physiology of mosquitoes in the last decades are summarized in a concise form. Such synthesis was badly needed in culicidology, — since a number of the original papers are unaccessible, the workers often have no time to spare to keep up with special literature, and frequently encounter linguistic difficulties.

For mosquito research workers, handbooks like MARTINI's Culicidae, BOYD's Introduction to Malariology, and BATES' Natural History of Mosquitoes, (beside taxonomic works), are quite indispensable. However, all these works became, in the course of time, obsolate. CLEMENTS book successfully fills the gap of the last decades.

Some of the 16 chapters of the book are more of a theoretical character, — though in close connection with practice, with the everyday work of culicidologists and malariologists.

The first chapter on *the egg* reviews the works about embryonary development, experimental embryology, genetic control of development, structure and properties of the egg shell, and ends with the mechanism of hatching.

In the chapter of *larval nutrition, excretion and respiration*, the anatomical structure of mouth parts, alimentary canal, pH of the larval gut, its enzymes and digestion are described. The diets on which healthy larvae can be produced under sterile conditions are given in Tables. Food reserves are stored mainly in the fat body, but also in other organs. The Malpighian tubes and the rectum serve for excretion. The tracheal system opens to the atmosphere, but some species make extensive use also of cutaneous respiration. The organs effectuating osmotic and ionic regulation of the body fluids are the anal papillae, Malpighian tubes, midgut and rectum. Most freshwater bodies are suitable for several species, but, due to a discrimination by ovipositing females, the number of species is restricted.

Temperature affects *growth and metamorphosis* of the larvae, pupae and the structure of imagos: size, number of follicules, color and wing length. A detailed description is given of the development of imaginal buds, the metamorphosis in the pupa and the maturation of the adult.

The *circulatory system* is rather simple and experiments suggest that the mosquito heart is of the non-innervated myogenic type.

The thermal limits of *survival and longevity* are influenced by a number of factors. Survival at low and high temperatures and the influence of acclimatization on its criteria are discussed.

From the examination of the organs and the mechanism of *adult feeding* it may be inferred that the chemoreceptors on the labella, on the tip of labrum and within the cibarial pump mediate the responses of mosquitoes to blood and sugar solutions. Much work has been done on the salivary glands and their secretions, their content of agglutinins and anticoagulins, and the causes of skin reactions of the host. *Adult nutrition and metabolism* are closely dependent on temperature and also on humidity. The nutritive value of various carbohydrates are given, together with nutritional requirements for reproduction. The amino acids, essential for egg production, are shown in a Table.

After a detailed description of the organs of *reproduction*, the growth of the egg follicle is discussed. Autogeny is now known to occur very widely in many genera. Ovarial development is controlled by gonadotrophic hormones, as has been proved experimentally. Ovulation and oviposition appear to be under nervous control. Fecundity, that is, the number of eggs, is proportional to the amount of blood-meal and the size of the female. Because mosquitoes

must feed twice to transmit pathogens, the determination of their reproductive history is of great importance.

The main *sensorial organs* of mosquitoes are the compound eyes, the antennae, and the chemoreceptors of the mouth parts and legs. The compound eyes are of the apposition type. Their responses to light of different wave-lengths, the attractiveness of differently coloured surfaces, the course of adaptation to darkness, had been studied by many authors. The antennae are anatomically and physiologically different in both sexes. The extremely developed second joint is filled with Johnston's organ. It serves as a hearing organ, sensible only to the frequency of the female's flight tone, — whereas that of the male is outside of its range — an observation in contradiction to the earlier belief that swarms of males are kept together by their own tunes. The flagellum has many different chemoreceptors, but their function is still to be disclosed. Contact chemoreceptors sensible to glucose are on the labellae and tarsi; those in the cibarial pump are sensitive to blood. Other sensorial organs are on the halteres, maxillar palps, thorax and wings.

Many unsolved problems arise from the phenomenon of *diapause*. There is a difference between quiescence, that ceases on amelioration of the environment, and diapause which is terminated only after reactivation by cold or other factors. In univoltine species, the eggs enter an obligatory diapause, whereas in multivoltine species, the diapause is facultative. The stimuli to egg hatching are submergence in water and low oxygen tension, but they also depend on conditioning. Evidence is insufficient to decide whether the overwintering larvae are simply quiescent and able to continue development at any time after a rise in temperature, or require an increase in day length. The hibernation of adults is found in most *Anopheles*, but is rare among aedine mosquitoes. Females which pass through gonotrophic cycles never subsequently enter hibernation. The induction of diapause is due to exposure of the developing insects to short day.

Behaviour of larvae and pupae can be analyzed into tactic and kinetic responses. Larvae swim along until they attain the preferred temperature zone. Under the influence of strong light, Anopheles messeae larvae swim and dive incessantly. Diving is also the only defense against predators and is stimulated by vibration and shade.

Control of adult activity depends mainly on the circadian rhythm, maintained by a physiological clock within the organism. This must be "set" by external stimuli, such as change from light to dark. Thereafter it can be independent of the environment. Circadian rhythm controls oviposition, biting activity, and flight activity which are, on the other hand, influenced by the microclimate: humidity, temperature, light. Low temperature, light and wind can inhibit activities, or they may incite them before time.

Flight behaviour was thoroughly investigated, and surrendered an insight into the mechanism of orientation of flight. Mosquitoes usually fly upwind, not tolerating images passing over their eyes from back to front, nor moving laterally. Downwind, they fly only high above the ground, — in this way they can travel great distances.

Host seeking behaviour of the mosquitoes depends on the phase of the gonotrophic cycle, the circadian rhythm, microclimate and responses to individual factors, such as body temperature, moisture, carbon dioxide, odour (sweat, blood, urine), and various visual factors of the host. Antennae are of great importance in the perception of air-borne factors; odour is important in attraction from a distance.

Reproductive behaviour is characterized by mating, swarming and ovipositing. Males are attracted to females not by odour, but by sound. Copulation is initiated during flight, but some species will mate also when at rest. Both males and females will copulate repeatedly, but males can inseminate only a small number of females. In overwintering females sperm must live for several months. Swarming of the males takes place over some markers which contrast with the background, e.g., trees, chimneys, steeples. Females are also attracted by markers, thus swarms concentrate a population with obvious advantages for mating. Other authors contend that swarming is just a consummatory act, the function of which is not known. Ovipositing females are attracted by sight, and by using chemoreceptors on the legs when examining water.

The book gives an up-to-date, comprehensive survey of the organism, physiology and behaviour of mosquitoes, and will be an indispensable guide for all workers in the field of pure and applied culicidology.

FERENC MIHÁLYI (Budapest)

Bio-Telemetry (The use of telemetry in animal behaviour and physiology in relation to ecological problems.) Proceedings of the Interdisciplinary Conference, New York, March 1962. Edited by L. E. SLATER.
Pergamon Press, Oxford (1963) £6/6/5.

The term telemetry recalls to almost everibody's mind notions like space flight, rockets, airforce, war, etc. It is quite a surprise to learn by reading this interesting book that the significance of biological telemetry, however closely related in its development to technology of space probes, is different and of a higher order. We learn that it may assure a general extension of human scope around our world. It may promote a more extensive knowledge of our natural environment and, as a consequence, may facilitate to plan properly the utilization of natural resources to cope with the problems arising from an ever increasing population. The highly humanistic atmosphere of the book is best exemplified by the quotation of one of its co-authors N. J. HOLTER who says: "I have a feeling that the materials to be discussed in the next four days . . . constitute a good advance toward understanding the animal world and are of such significance that they could eventually be a prominent factor in solving the greatest problem in history — that of creating world peace." It is evident that the realization of such a program calls for bold and imaginative thinking and the utilization of modern research techniques requiring the permanent co-operation of both biologists and physical scientists.

The book "Bio-telemetry" consists of the documents of an interdisciplinary conference organized in 1962 in the American Museum of Natural History in New York, with the participation of more than 50 scientists representing in a sound equilibrium both biological and physical sciences. The original reports were divided into the following six sessions: (1) The potential for telemetry in biological research, (2) The current state of biological telemetry, (3) Reports on telemetry in animal tracking, orientation and ecology, (4) Reports on telemetry in studies of animal social behaviour, (5) Reports on telemetry in studies of animal physiology, (6) Reports of telemetry in studies of physiology in man.

These 6 sessions cover almost completely the fields where the use of telemetrical methods seems or proved to be succesful. Every paper is followed by a stimulating discussion, which gives a clear insight into the working method of the conference, moreover it facilitates the critical evaluation of the material presented.

The first session deals with general problems, limits and applicability of telemetry in biology. We get sober warnings not to abuse telemetry when direct observation or other simple means are helpful. We learn that telemetry is a special branch of science which can only be succesfully cultivated with a close co-operation of biologists and physicists. Special problems arise by the need to create common basis for understanding. Both sciences use highly specific languages and MCCULLOCH, one of the pioneers in the coupling of the two sciences reminds the participants of the importance to learn to talk to one another. From this point of view the papers presented are exemplary on both sides. They do not pass the limits by presenting data where they would overcomplicate the picture and hinder common understanding. References are given after each paper.

The second session is particularly interesting for biologists because in this section competent experts of telemetry technologies give details concerning new achievements. It appears that industry produces a great variety of aerospace hardware, available off-the shelf, unfortunately often surpassing the financial abilities available for biological research. The functional capacities of these telemetry systems generally offer even more than required by present day biological problems. Both adaptation of these complex systems and constructions of new and simpler devices encounter, however, economical and commercial difficulties. It is hard to find companies which could afford to invest into the development of new biological telemetring componenets in view of the unstable and relatively small demand. That is the point where a well organized and continuous financial support from special or government might help.

The most serious requirements of biological telemetry appear in the field of miniaturization. The second section of the book offers a lot of information concerning new achievements in different parts of telemetring systems, transducers, transmitters, antennas, etc. The production of micro-electronic technology — e.g. the high density packing techniques, integrated circuits, functional block or molecular circuits — are really amazing and at the same time very promising also for the field of microminiaturization of bio-medical telemetring circuits.

The third session of the conference deals with special biological reports. It gives an account of special experiments carried out with the help of elaborated telemetering techniques in various animals. (Orientation and homing of birds, radio tracking of grizzly bears, telemetric monitoring of woodchuck movements, travel of marine turtles, fish homing and similar ecological problems.) The biological informations are always kept between the limits of their

technological relations, according to the special purposes of the conference. They are, however, highly interesting to the biologist because of the simultaneously demonstrated and critically discussed technical solutions of these biological problems.

The fourth session discusses the applicability of telemetry to studies of animal social behavior. Some interesting achievements in both biological and technical respects are presented moreover evidences yielded that telemetrical methods give unique possibilities in the scientific approach of animal group processes.

The fifth session deals with telemetric registration of more elementary physiological events (blood pressure, EEG, ECG, temperature, etc.). Some interesting problems of recording physiological changes during the conditions of space-flight are also presented.

In the sixth session application of telemetric techniques to special human problems (ingested or subepidermally implanted sensors) moreover questions of instrumentation for the acquisition and reduction of data from extended periods of time are analyzed.

On the whole the book represents an excellent guide for both experts and beginners. It gives a general survey of a field which is still in early development which, however, thanks to the new technical requirements of space probes evolves extremely rapidly. It is the chief merit of the book that it calls attention to the fact that the importance of telemetry goes far beyond the goals of space-flight. It gives convincing evidences that its most important perspectives lay in the analyses of the many faceted phenomena of biology. The content of the book even beyond straight expression convinces that this program needs a broader social understanding and more efficient support than obtained at present.

ENDRE GRASTYÁN (Pécs)

F. B. SALISBURY: **The Flowering Process**
Pergamon Press, Oxford (1963), pp. 234, 50 s.

The aim of the present work of Dr. F. B. SALISBURY, professor of plant physiology at the Colorado State University, was to discuss the physiological processes of the conversion from vegetative to reproductive state in higher plants. According to the author the book is addressed to the graduate students and others interested in the topic "at the graduate level". Undoubtedly, the book is not a comprehensive treatment of the physiology of flowering covering the whole field. Still I have the feeling that the author underestimated the prospective role of this book in the plant physiological literature. In my opinion research workers will also greatly benefit from the ideas and from the clear-cut presentation given by Dr. SALISBURY who having authority based on first-hand experience handles the topic with such an inspiration that can come only to those fighting in the first line to acquire new knowledge. This is particularly evident in the second half of the book which is written relying heavily upon personal experience.

The introductory chapter provides the reader with a "biological framework" of the flowering process. It is followed by chapters on the response types, on the ecology of flowering, on the role of low temperature, on methodical problems, on the role and nature of the absorbing pigment, on the problem of timing, on the synthesis of flowering hormone and its mode of action.

Selected bibliography is given. The selection was perhaps somewhat too rigorous. Altogether 81 papers are referred to.

The book is attractively produced. The illustrations are good. In addition to illustrations supplied by other authors there are quite a few which have never been published before. The price is reasonable.

In conclusion it may be stated that the appearance of the book which is the 4th volume of the Plant Physiology Division of the International Series of Monographs on Pure and Applied Biology will be welcome by plant physiologists all over the world.

G. L. FARKAS (Budapest)

Animal orientation (Orientierung der Tiere)
Symposium in Garmisch-Partenkirchen 17—21. 9. 1962. *Ergebnisse der Biologie*, **26.**
Springer Verlag, Berlin (1963), pp. 313. DM 50.—

In September 1962 in Garmisch-Partenkirchen (West-Germany) an international symposium devoted to the problems of animal orientation-mechanisms took place. The symposium was initiated by Professor HENRI PIÉRON and a great number of illustrious biologists took part in its work. This volume includes the papers read at the symposium.

The analysis of animal orientation is a central problem of modern experimental zoology. The new trends in neurophysiology, namely the electrophysiological, biophysiological and mathematical approach make the solution of many unsolved problems of "instrinctive" orientation possible. Consequently the questions of animal orientation are mainly physiological problems of the special senses and of the central nervous system. It is therefore not by chance that 18 of the total 25 papers of this volume deal with neurophysiological analysis of orientative behaviour. The orientation-mechanisms of various invertebrates (chiefly of arthropodes) as well as of vertebrates (fishes, amphibians, birds, etc.) is treated in detail.

Although the documentary presentation of free discussion, wich apparently followed each lecture, would undoubtedly have made this book more interesting, the rich material of the 25 papers is an important contribution to modern experimental biology.

This book is recommended to those interested in experimental zoological, neurophysiological and psychological problems concerning the mechanism of special senses and instinctive behaviour.

G. ÁDÁM (Budapest)

Chemical and Biological Aspects of Pyridoxal Catalysis
Edited by E. E. SNELL, P. M. FASELLA, A. E. BRAUNSTEIN and A. ROSSI-FANELLI
Pergamon Press, Oxford, (1963), pp. 599. 60 s.

In October, 1962, an international symposium was held at Rome as part of the scientific program of the International Union of Biochemistry. This book contains the original papers presented and also their discussion. As indicated by the title of the volume, the contributions cover a wide field extending from the exact chemical formulation of pyridoxal catalyzed non-enzymic reactions to the hormonal regulation of pyridoxal enzymes in the intact organism. The volume as a whole gives an excellent survey of not only the results obtained so far with the different approaches but also of the unsolved problems, and the current ideas of the world's experts on pyridoxal catalysis.

Of the 48 papers presented the first eight contributions are dealing with the investigation of non-enzymic reactions catalyzed by pyridoxal derivatives in aqueous solution. In his opening address SNELL summarizes the conclusions reached from the study of simple model systems, which was initiated in his laboratory almost 20 years ago. The papers presented from the laboratories of MARTELL, JENCKS and METZLER are mainly concerned with studies of the kinetics of non-enzymic reactions of pyridoxal analogues. JENCKS and CORDES elaborate their view that transaminase apoenzymes should be expected to form intermediate Shiff bases with pyridoxal phosphate (PLP) in which way the catalytic potential of PLP can be greatly enhanced. BRUICE and TOPPING give an account of studies on the catalytic role played by buffer ions in model systems which favour the view that pyridoxal catalyzed transamination is subject to general acid-base catalysis. There is a very vivid discussion of the quantum mechanical considerations of pyridoxal catalyzed reactions presented by PULLMAN.

Due to the recently achieved high purification in quantity of the aspartate-glutamate transaminase from pig heart, a considerable part of the following papers is concerned with detailed investigations of the properties of this enzyme. The studies described by SIZER and JENKINS are dealing with a spectrophotometric investigation to characterize and determine the possible sequence of molecular species of enzyme bound pyridoxal phosphate in the course of transamination. Further papers refer to the primary structure (TURANO et al., FISCHER et al.) and resolution of the same enzyme. Studies are reported on isozymes, and HOLZER and SCHREIBER describe an analytical method for the simultaneous determination of microgram quantities of pyridoxal phosphate and pyridoxamine phosphate by using the purified apoenzyme of glutamate aspartate transaminase obtained from baker's yeast. An important refinement of the analytical procedure for tracing the aspartate — oxaloacetate transformation is reported from VERNON's laboratory.

Mainly based on the use of glutamate aspartate transaminase, detailed studies are reported on the mechanism of inhibitation of pyridoxal enzymes by a great number of agents (MARDASHEV, MAKINO et al., CONNARD, KHOMUTOV et al., KARPEYSKI et al., MUSAJO et al., CEDRANGOLO, MAKINO et al.). Quite a number of new antagonists are discussed. Great interest is attached to the recent claim made by CONNARD, according to which some derivatives of PLP, substituted in the carbonyl group like the semicarbazide or hydrazide, might function as coenzyme in PLP dependent enzymatic reactions.

In comparison to glutamate aspartate aminostransferase other PLP enzymes are less thoroughly treated. There is one paper on the properties and metabolic role of each amino-

malonate decarboxylase (UDENFRIEND), cysthathionase (CAVALLINI et al.), threonine dehydrase (HAYAISHI et al.), and tryptophanease (HAPPOLD and SCOTT). Both CATTANEA and SENEZ, and MEISTER et al. investigated the role of loosely bound PLP and/or α-keto acids in the function of β-aspartate decarboxylase, which enzyme has long been known to have this unique requirement. Detailed investigations of diamine oxidases are described by DE MARCO, MONDOVI et al., GORYACHENKOVA and ERSHOVA, and KAPELLER-ADLER. A finding which concerns the apparently related plasma amine oxidase is presented by YAMADA and YASUNOBU. The demonstration of the occurrence of a copper-chelated and firmly protein-bound derivative of vitamin B_6 in plasma amine oxidase which is reported from YASUNOBU's laboratory is certainly one of the most important new results discussed at the Symposium.

Finally, some further papers are discussing special topics. To mention only a few of these, the participation of PLP enzymes in microbial transsulfuration reactions is discussed by FLAVIN, while FISCHER et al. are dealing with the properties and role of PLP in phosphorylase. The possibility of the participation of PLP in the penetration into and accumulation in living structures of amino acids is treated by CHRISTENSEN.

The symposium which opens with the address of one of the leading figures in this field, Professor E. E. SNELL (California), is closed with the paper of the other outstanding expert of pyridoxal catalysis, Professor A. E. BRAUNSTEIN (Moscow). BRAUNSTEIN's paper on current progress in research on biological pyridoxal catalysis gives a concise survey of both results and challenging problems in this field undoubtedly stimulating further progress, as does the volume as a whole.

JÁNOS SÜDI (Budapest)

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