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REGENERATION AND WOUND HEALING

AKADÉMIAI KIADÓ, BUDAPEST

REGENERATION AND WOUND HEALING

Symposium in Budapest November 1960

Edited by GY. SZÁNTÓ

(Symposia Biologica Hungarica 3.)

Bulgarian, Hungarian, Polish and Russian scientists contributed to this Symposium on regeneration and the biological mechanism of wound healing. The papers deal with the relations of regeneration and somatic embryogenesis, biochemical and histochemical problems of wound healing and regeneration, and the connections between regeneration and the growth of tumours.

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PREFACE

This volume presents the lectures delivered and the accompanying discussions at the Symposium on Wound Healing held on the 8th and 9th of November, 1960 in Budapest.

First of all our thanks are due to the Hungarian Academy of Sciences for sponsoring this scientific meeting.

The subject of the Symposium was the discussion of the biological and biochemical mechanisms underlying wound healing. Naturally there could have been no claim to cover the extremely wide field. The main object of the meeting was to bring together in free discussion research workers of various disciplines; surgeons, biologists, biochemists, experimental workers and clinicians. The problem is of such multilateral complexity that completeness was out of question from the start. All over the world a great number of investigators are conducting researches at various levels pertaining to wound healing. We suppose that the talks held without any claim to completeness were useful nevertheless and the participants gained some useful stimulation for their future research.

It is a pleasant duty to express our thanks to the chairmen of the Symposium, Academicians B. Kellner, F. B. Straub and J. Szentágothai, for directing the discussion to the advantage of all participants.

The object of publishing the proceedings is not only to record the work of the Symposium, but to bring the results to a wider audience for possible use. From this respect it is regrettable that owing to technical and organizatory difficulties the proceedings appear so delayed.

Finally we wish to express our thanks to all who contributed to the recording of the discussions and helped to edit this volume.

G. Szántó

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REGENERATION AND SOMATIC EMBRYOGENESIS

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Synopsis

The author suggests a distinction between phenomena of regeneration *(i.e.* new-formation of the lost parts of the organisms) and somatic embryogenesis *(i.e.* the development of whole organisms from somatic cells). The somatic embryogenesis is always preceded by a disintegration of the normal correlations in the cellular systems, the tissues, the organs or within the whole organism. On the contrary, the processes of regeneration cannot normally proceed when the organism or the remnant part of an organ becomes disintegrated. Organisms having a low degree of integration *(e.g.* plants, sponges, lower Coelenterates, lower worms) are generally capable of reproducing themselves asexually and producing phenomena of somatic embryogenesis. In spite of a generally accepted opinion, plants, sponges and lower Coelenterates have a weaker regenerative capacity than organisms with a more complex organization and with a higher degree of integration. According to the author, asexual reproduction is always accompanied by the disturbance of normal integration in the organism. On the basis of his experiments the author states that within the group of Coelenterata, with the organization becoming gradually more complicated, the capacity for asexual reproduction and somatic embryogenesis is decreasing more and more, whilst the regenerative capacity shows an upward tendency. For example, the Ctenophora *(e.g.* the species *Bolinopsis infundibulum* and *Beroe cucumis)* regenerate very well. During the ageing of organisms, *i.e.* with the gradual weakening of the various integrating mechanisms controlling the life of the organism as a whole, regenerative capacity will gradually be lost. The well-known hypothesis according to which the regenerative capacity of organisms decreases in the course of evolution must be regarded as unfounded. It is not the regenerative capacity which is lost as the organization becomes more complicated and the level of integration higher, but the capacity for asexual reproduction and somatic embryogenesis. On the contrary, phenomena of regeneration do not lose their importance in the course of evolution but they show progressive tendencies and assume specific features in various groups of the animal kingdom. The author discusses the evolution of regeneration phenomena and somatic embryogenesis. He emphasizes, that reparative regeneration originated and developed on the basis of the physiological regeneration, that asexual reproduction and regeneration have a different origin and they could not develop simultaneously in the course of evolution.

Introduction

The existing classification of regeneration phenomena cannot be regarded as acceptable. In our papers [see 30] we have dealt with this problem and we think the use of the single term "regeneration" for the various morphogenetic processes occurring in the plant and animal kingdom to be forced and unjustifiable. The following processes are termed *e.g.* "regeneration": the restitution of the lost foot of the hydra as well as the development of a whole hydra from

a fragment presenting only $^1/_{200}$ part of its body; the development of new sponge individuals after having squeezed sponges through a fine-meshed cloth; the restitution of the amputated foot of the triton; the healing of skin wounds in man; the development of whole plant organisms from a single somatic cell or from a group of such cells.

Let us use our scientific imagination: biology will render possible the asexual reproduction of the mammals, *e.g.* the development of such an animal from epithelial cells of the skin. According to the phenomena mentioned above, this process would also be termed as "regeneration" similar to the wound-healing processes of the skin. Since regeneration can be considered a reasonable term, the development of the whole organism of *Begonia rex* from somatic cells of its leaf, as well as the wound-healing processes of its organ provoked by injuries is feasible. Biology at Trembley's age, *i.e.* in the 18th century, was contented with the single term "regeneration" for the designation of various phenomena, as we suppose was medicine of the same period which gave a single name, "lung disease", to numerous pathologic processes *e.g.* the inflammation or the cancer of the lungs. However, when it is necessary to clarify the causes of the origin of phenomena as well as the laws of their course, a too wide application of this term will necessarily inhibit a thorough analysis.

It is not accidental that most workers, using the concept of "regeneration" in a wide sense of the term, have been forced to introduce auxiliary terms for certain categories of the phenomenon ("morphallaxis", "restitution", "heteromorphosis", "multipolar forms", etc.). As it will be seen later, the appearance of such auxiliary terms points to fundamental differences between the various phenomena generally called regeneration.

The morpho-physiological term "regulation" is often used. It is also applied for such a large group of phenomena that its actual meaning is disappearing and, therefore, it cannot help in scientific work. The term "regulation" is generally used for the capacity of the organism to maintain and, after being disturbed, to re-establish the properties of its physiological processes and structures. It is evident that this meaning has a bearing on the greater half of all physiological and morphological processes taking place in the organism.

It is possible, however, that the term "regulation" should be reserved for the designation of certain re-establishing processes of the normal embryogenesis occurring after injuries of various "anlagen" of the embryo *(e.g.* the developing medullar plate or tail-bud of the amphibian embryo). In the regulation of normal development, the term of regeneration or other similar terms cannot be applied in view of their use for the designation of re-establishing processes of fully developed organs. For example, it would not be right to speak about the regeneration of the amphibian tail in the state of the tail-bud when the tail does not really exist.

The terms "physiological regeneration" and "reparative regeneration" being generally accepted, are undoubtedly very important, however, they are not interpreted unanimously. With the term "physiological regeneration" one should designate processes during which functioning tissues and organs will be renewed (*e.g.* the continuous substitution of the superficial layer of the epidermis, the re-establishment of gland structures being destroyed during function; hair and feather moulting, etc.).

In order to give a new and more reasonable classification of the "regenerative phenomena", one should, first of all, separate the processes of the true

regeneration from that having quite a different genesis and morphogenetic character. In this sense, developmental processes resulting in the formation of whole organisms from a single somatic cell or from a group of such cells, might not be regarded as regeneration. These phenomena should be designated as *somatic embryogenesis*, in contrast to the *true regeneration* processes during which the organisms are developing their lost body-parts. The phenomena of the true regeneration require, of course, a special classification. The following processes can be regarded as regeneration phenomena: the restoration of lost parts in *Stentor* (Ciliata) into new organisms; the wound-healing processes of the sponges; the new-formation of the foot and the hypostome with the tentacle in hydras, the development of the amputated extremity of an urodelan; the healing of wounds formed on the leafs of *Begonia*, etc.

The observations of the author and his co-workers were summed up in a monograph entitled "Regeneration and somatic embryogenesis" [30]. The following is a brief extract from the author's book: "Far from presuming to have solved the discussed problems of regeneration the author hopes that this book could be useful in furnishing material for the discussion of this problem of great theoretical and practical importance" [30, p. 3].

In this paper we confine ourselves to give a brief account of the most important facts and hypotheses. It is to be regretted that owing to a limited space our paper does not allow us to give discussion adequate to the complexity of the biologic phenomena in question. By formulating one or other regularity relevant to regenerative phenomena, strict rules cannot be adhered to. Any idea might generally be brought to an absurdity. Investigators in any scientific field, after having made a statement often add a "but". Their doubts show that within the animal kingdom, where evolution has produced so many types of organization, the basic tendencies could not develop in one direction and we find many deviations from the general rule. This applies, naturally, not only to the phenomena of regeneration and somatic embryogenesis, but also to the evolution of all biological characteristics which make the demonstration of the basic tendencies more difficult. Regeneration had its evolution in the course of evolution of the living world and it would be unnatural to apply the same principles to *Euglena* and *Begonia* or to sponges and man.

However, could this mean that one must despair of formulating new rules?

The main differences between regeneration and somatic embryogenesis

Somatic embryogenesis is always preceded by disintegrating processes altering the normal correlations existing between the cells, tissues and organs or within the whole organism. This disintegration is the main biological precondition for the development of somatic cells or cell groups into whole organisms. The new-formation of lost parts of the organism, the typical building up of the specialized tissues and organs as integrating body-constituents cannot proceed normally when the organism or the remnant part of an organ becomes totally disintegrated. Consequently, the various phenomena in question cannot be classified according to the size of the los parts, but according to the main differences of their morphogenetic processes.

Let us mention several preliminary examples.

After isolating the stem of the *Hydra oligactis*, we cut off its basal part. If performing the experiment we do not induce great injuries disintegrating the whole system of the isolated stem, *i.e.* if its original morpho-physiological system remains intact, the regeneration of a new basal part *(i.e.* of a new foot) can be observed after 24 hours. Under special experimental conditions, after having strongly disorganized the system of cells, *e.g.* by cutting off large parts (*e.g.* the whole gastral region) from the body of the hydra, no regeneration takes place, the oral and aboral pole of the body will not be replaced. In such cases, the part cut out from the body will be completely re-organized and will produce a whole organism *i.e.* somatic embryogenesis will take place.

Let us give another example. One cannot expect the extremity of an axolotl to be regenerated after having amputated it with its girdle or after having strongly damaged the stump of the extremity in some way or other. The tail of a newt will not be regenerated when cutting it off at the cloacal region, that is if no stump remains.

Numerous examples can be mentioned which demonstrate the role of the remnant parts *(e.g.* stumps) in the regeneration and showing, at the same time, the importance of local histogenetic processes taking place during the regeneration. In certain experiments, the tail and the extremity still capable of regeneration of anuran larvae have been transplanted on the back or on the flank of an adult frog, then, after adnation they have been amputated. Afterwards, the parts cut off were regenerated in spite of the known fact that the adult anuran has no capacity of regenerating the extremities. All these examples clearly demonstrate the importance of the morpho-physiological system of the stump of the regenerating organs.

After having reduced to its half the wound-surface made at about the level of the knee-joint on the proximal region of the axolotl's leg [20], a defective regeneration takes place: only the tibia and a finger regenerate (Fig. 1 *A*). The cutting off of the extremity at any level, including the most distal one, does not lead to complete regeneration: the structure of the foot and the fingers shows atypical (Fig. 1 *B* and *C*).

In our laboratory, we have irradiated with locally X-rays (8000 r) the right hind extremity of the Siberian newt *(Hynobius keyserlingii)* in a 5—7 mm wide region of the thigh as far as the knee-joint. The left hind extremity remained untreated. In the control sets, the whole distal part of the extremity has been treated as far as the knee-joint. These control animals served to demonstrate whether the irradiation inhibited the regeneration or not. On the 5th day after treatment we amputated both hind extremities at the distal region of the shank so that on the stump of the experimental animals an area of 4—5 mm was left unirradiated (Fig. 2 *A*). It is known that 8000 r irradiation inhibits regeneration for a long time.

Our experiment has led to the following results. The control animals regenerated their left unirradiated extremity. As to the experimental animals, the right extremity started to regenerate (Fig. 2 *B*) similar to the left one which was not irradiated (Fig. 2 *B*). The unirradiated small disk of tissues played the decisive role in this case.

It is known that the extremity of an axolotl amputated at the thigh region does not regenerate with the missing part regenerating first and the leg and foot afterwards. The differentiation of the fingers at the distal pole of

the extremity starts prior to the regeneration of the thigh and foot. This interesting but not unique fact has not been duly considered till now although it represents the kernel of very important problems the solution of which requires entirely new, one may say revolutionary, hypotheses and experiments. What should be considered first is that morphological and histological dif-

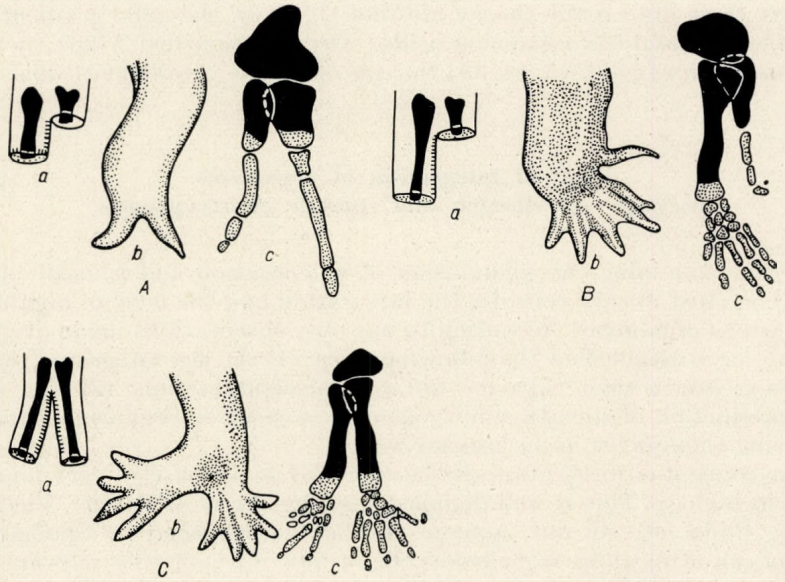

Fig. 1. Defective regeneration of the extremity of axolotl in different types of amputation of the leg: *A*—Regeneration after amputation at the proximal region; *B*—Regeneration after amputation at a more distal level; *C* — Regeneration after amputation at a still more distal region. *a* — scheme of the operation; *b* — the regeneratum; *c* — skeleton of the regenerated extremity; old skeletal parts are blackened [20]

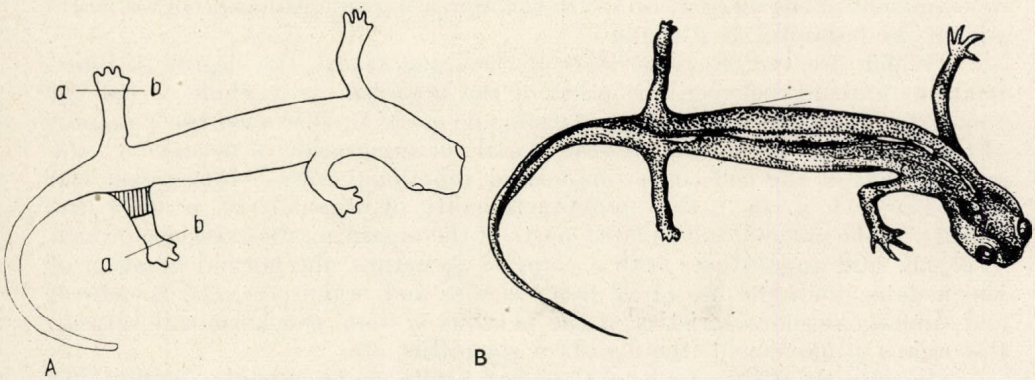

Fig. 2. Regeneration of extremity of *Hynobius keyserlingii*. *A* — scheme of operation; *B* — the newt with regenerating extremities; the part of the right extremity (lined) has been treated with X-rays. *a—b* — the level of amputation having left a *disk of tissues* without X-ray treatment (after Zdruikovskaia)

ferentiation are not necessarily simultaneous. On the whole it seems that most problems of regeneration cannot be solved at the level of cellular organization.

It is very likely that a number of structural laws of cell and tissue systems whose morphological manifestations include the organization of cells into tissues are still unknown. Scientists searched for these unknown laws in various directions. Such are *e.g.* the theory of Child [7] about the axial gradients, Spemann's theory about the organizing fields ("Organisationsfeld") [26], the theory of the "embryonic fields" [5, 13, 33], the concept of the "gradient-systems" [14].

Level of integration of organisms, asexual reproduction and somatic embryogenesis

When examining the phenomena of regeneration and somatic embryogenesis, one must always consider the integration and the level of organization of the various organisms. According to our own observations made over many years, we have established the following rules: (1) the less integrated the organisms the greater is their capacity for asexual reproduction; (2) the experimental production of somatic embryogenesis is easier in organisms capable of reproducing themselves in an asexual way.

The concept of integration can be regarded as the basis of very important theories in biology. This is well demonstrated by works of Pavlov, Viedensky, Zavarzin, Child, etc. In our opinion, the facts established by Spemann and his school can after all be regarded as the results of hypotheses relevant to the integration of the developing organism [26]. Dogiel [10] pointed out that the evolution and specialization of the various groups of Metazoa is accompanied by a gradual decrease in the number of the homologous organs and that, in certain cases, this leads to the intensification of the functions and the development of polyfunctional organs. This "concentration" of the organs which means an "oligomerization" compared with the totality of the organism, leads to the development of the integration *i.e.* to the increasing subordination of the parts within the organism as a whole.

Which are the characteristics of the integration? The degree of interrelations existing between the parts of the organism as a whole or, on the contrary, the degree of autonomy of the single parts; furthermore, the character of the functional specialization of cells and the integration of tissues *i.e.* "the organization of the cells into complicated, functional units — into organs and apparatus" [4, p. 367]; the "polyfunctionality of organs" [10, p. 327]; the vitality of the cut out and isolated parts of the organism, the existence or lack of organs and apparatuses with a complex structure the normal function of which determines the life of all other tissues and organs, *i.e.* the structural and functional characteristics of the nervous system, the hormonal system, the organs of movement, the digestive apparatus, etc.

In animals with a high level of organization one cannot possibly find organs having merely a single function. All organs are polyfunctional and the normal organism represents an integral correlative whole so that both the anatomist and the physiologist recognize the convention of the expressions: "character" or "organ"; indeed, it is impossible to speak about the

human heart without mentioning the aorta, or treat the eye disregarding the optic nerve.

Let us see now, which are the organisms characterized by the capacity for asexual reproduction and somatic embryogenesis. There is no need to emphasize that plants are less integrated organisms than animals. Primarily, the regulatory mechanisms characterizing most of the animals *(i.e.* the nervous system, the hormon-producing system) do not exist in plants.

The problem relating to the individuality of the plants is excessively complicated. Many botanists regard the leaf of a plant as an individual and

Fig. 3. Different cases of sprout-development. *a* — leaf of *Bryophyllum calycinum*; *b* and *c* — leaves of *Torenia asiatica*; *d* — formation of callus on an internodal section of a dicotyledonous plant and the consecutive development of sprouts; *e* — *Veronica annagalis* (5/9 of the natural size): isolated inflorescence cultivated for three months with striken roots

a plant *(e.g.* a birch-tree) as a colony composed of many thousands of such "individuals". The low degree of organization is responsible for the various forms of asexual reproduction wide-spread in the plant kingdom. For example, new sprouts can develop from roots, leaves, stems, flowers and from the callus developing on the wound-surfaces. A general way of asexual reproduction is the fragmentation of the parent organism into parts developing then into new individuals *(Marchantia*, the fern species *Salvinia* and *Azolea)*; the species *Bryophyllum, Torenia asiatica*, species of the fern genus *Asplenium* and many other plants reproduce themselves through their detached pinnated leaves (Fig. 3). It would be very interesting to investigate the processes taking place here according to our hypothesis *i.e.* supposing that interrelations exist between the level of integration and somatic embryogenesis. A preliminary condition to these processes *(e.g.* the formation of sprouts from the tissues of the callus) as to somatic embryogenesis provoked experimentally in sponges and hydras, is the local disintegration of the tissues.

The phenomena of true regeneration generally cannot be found here in such a form as in animals; this is another fact connected with the low integration of plant organisms. It is known, *e.g.* that the parts cut out from the leaves of any plant will not regenerate. The processes taking place on the wound surface and having rather immunological than morphogenetic character will not be

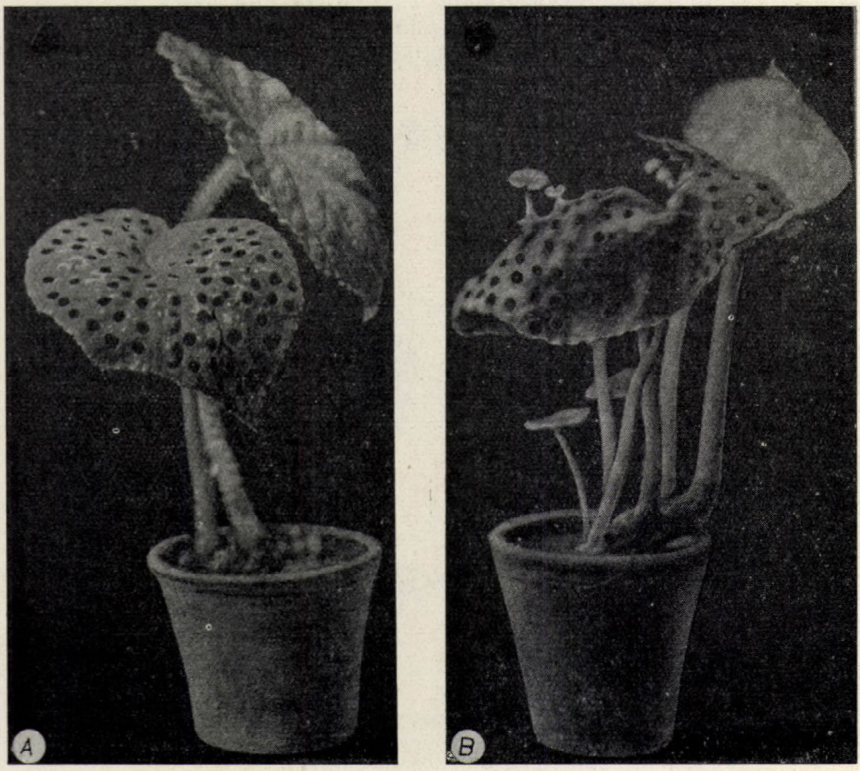

Fig. 4. Development of sprouts on the leaf of *Begonia rex* left in the parent plant. *A* — leaf transformed into a "sieve"; *B* — formation of sprouts [12]

dealt with. As shown by the objective evaluation of the literary data, many authors succeeded in provoking, as exceptional cases, processes similar to the regeneration phenomena of animals. As to the identification of these processes, special investigations are required before one can speak of any degree of identity. In very young tissues, *e.g.* in the embryonic regions of roots or in the prothallium of ferns "regenerative processes" for the rejuvenation of the organs can be provoked. These phenomena, however, are more related to regulation processes observable in disturbed embryonic development.

Consequently, it is wrong to apply the term "regeneration" to phenomena of somatic embryogenesis which are very frequent in the plant kingdom. Plants having a low degree of integration do not have greater capacities than that of true regeneration.

In connection with the problems exposed above, Girfanowa and Tokin [12] performed the following experiment. In one or two leaves of *Begonia rex* by using a metallic tube, they punched several holes of a 3—5 mm diameter close to each other (Fig. 4 *A*). The operated plants were kept in cultivating cases in a wet atmosphere at 20—25° C. The experiment has been performed on

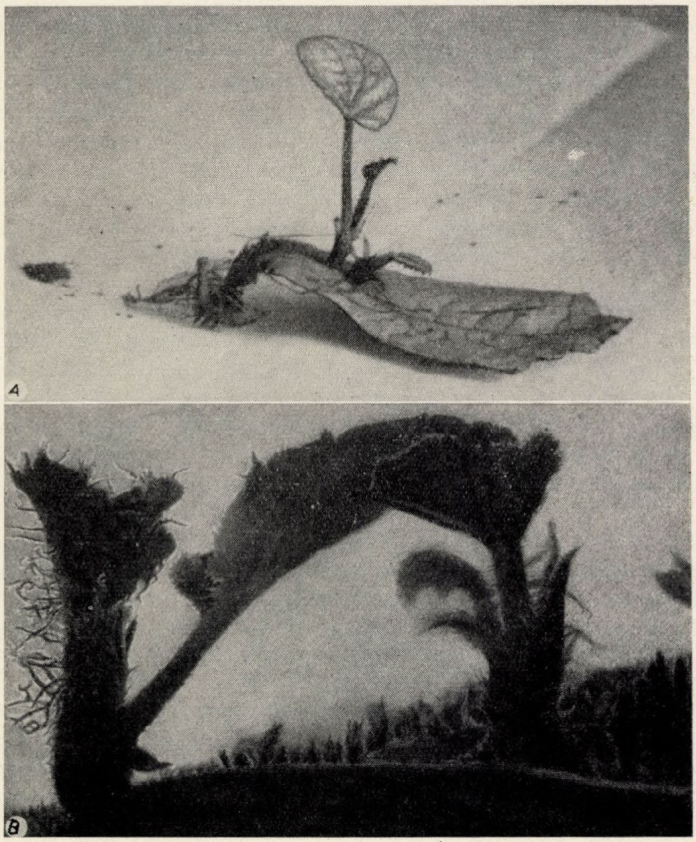

Fig. 5. Formation of sprouts on the leaves of *Begonia rex* [36]

48 leaves. In the course of a month, most of the operated leaves died and fell off. During this period, however, 16 plants developed roots on their operated leaves, at the borders of the excisions and after a week the first accessory leaves appeared in certain places of 10 operated leaves (Fig. 4 *B*).

In another experiment they cut 28 adult leaves of *Begonia rex* into equal halves and incised one half with a saw-toothed scalpel thus inducing a strong disintegration of the tissues; the other half of the leaves was incised with a sharp razor. Both halves of all leaves were then placed into a box containing wet sand at 16—22° C. All experimental leaf-sections *(i.e.* which had been incised with a saw-toothed scalpel) developed new-formations after 14—15

days, whilst the control ones (being incised with a sharp razor) developed accessory sprouts after 17—18 days (in 27 cases out of 28).

The data of Zawadsky [36] support our statements. New-formations can be provoked on old leaves, too, with the method described above *(i.e.* by perforating the leaves). This can be explained with the connections existing between somatic embryogenesis and the integration of tissues, the organs of the organism. In his experiment Zawadsky cut off all sprouts and leaves from the stem of *Begonia* with the exception of a single adult leaf. This experimental series on plants having originally 4—12 leaves resulted in a single adult leaf of *Begonia*, developing many new sprouts without any previous injury (Fig. 5 *A*, *B*). One of the leaves having a surface of 180 cm^2 developed 5700 sprouts!

One can succeed in helping also the miniature sprouts to strike root.

According to Zawadsky, under the conditions of these experiments a general disintegration of the tissue of the leaf took place which ceased the integrity of the leaf. As a consequence, the cells of the epidermis after escaping the normal tissue connections transformed into meristemal cells and developed sprouts.

Other botanists have also observed interesting examples of somatic embryogenesis. The infection with *Bacterium tumefaciens* provokes tumours within the tissues of the potato and simultaneously the formation of numerous sprouts can be observed. Considering that the unique morphogenetic role of this bacterium consists in producing anarchy in normal tissues and destroying the normal morpho-physiological correlations, the development of sprouts in these interesting experiments can well be understood on the basis of our hypothesis.

In the course of the evolution of the animal kingdom the capacity for asexual reproduction (as well as the possibility for provoking somatic embryogenesis experimentally) decreased with the gradual development of animal organization. It is a fact that the lowest level of integration in the group of Metazoa is represented by sponges and coelenterates. Accordingly, these animals are generally capable of asexual reproduction and phenomena of somatic embryogenesis can be easily provoked experimentally. The evolution of integration can naturally be found, to a certain extent, within the phylum of the sponges, too. According to this, the phenomena of regeneration and somatic embryogenesis are different in the various sponge species. This interesting problem is investigated in our laboratory by Korotkova and Volkova [17, 18].

Every sponge species has the capacity for asexual reproduction by budding, fragmentation or by forming gemmules and sorites. In the group of sponges the asexual form of reproduction dominates. According to most recent data, numerous marine sponge species also reproduce themselves in this way, *e.g. Suberites domuncula*, the genus *Haliclona* from the family *Haploscleridae* etc. In the group of Calcareous sponges *(e.g.* in the genus *Oscarella)* the isolation of certain body parts leads to the formation of "buds". In other sponge species *(Donatia lyncurium* and certain representatives of the *Tetraxonida)* there are external buds consisting of a cell-aggregation (archaeocytes) similar to the gemmules. The sorites represent compact syncytial masses composed by archaeocytes. The formation of sorites can be found in the species of the orders *Triaxonida*, *Tetraxonida* and *Cornacuspongida* as well as in that of the family *Lubomirskiidae* living in the Baikal lake. In the sorites, the embryo

probably develops from a single cell consuming the other part of the syncytial mass. In this way a free swimming larva will develop which resembles the sexual development. One should suppose that in budding a local disintegration of the sponge body takes place, whilst in the formation of sorites and, first of all, gemmulae this disintegration is more extensive.

In the fresh-water sponge genera *Spongilla* and *Ephydatia* as well as in the marine ones *Suberites* and *Ficulina* unfavourable conditions bring about a disintegration disorganizing the body of the animals. Large parts of the sponge body will gradually decompose and in various areas gemmulae will develop. During these processes certain cell types will be destroyed, the phago-

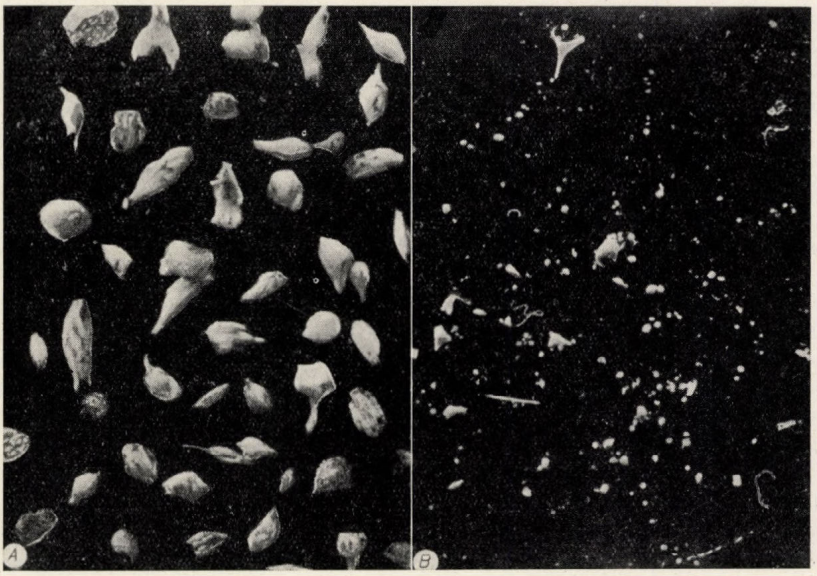

Fig. 6. The sponge *Geodia barretti*. *A* — young sponges developed from regenerative bodies and collected from the sea; *B* — regenerating cell masses after squeezing the sponge [6]

cytic activity intensifies, etc., so that the body of the sponge seems to be formed by irregularly dispersed aggregations of unicellular constituents. The gemmulae formed survive to the body of their parents and will develop, at the beginning of the next spring, into new multicellular organisms. The latter leave the parental body through the pores of the gemmulae and develop into new sponge individuals.

Burton [6] describes a vegetative reproduction in several marine sponge species, including the following remarkable case. On the sandy beach he found many thousands of body fragments of *Halichondria boverbanki*. No signs of their adhesion could be found and most of them had drifted out to the sea. Investigations proved that these "regenerating" fragments originated from sponges the body of which had been broken up by the surf. It is possible that these phenomena also represent a form of vegetative reproduction.

The same author made other interesting observations and experiments also on the sponge species *Geodia eoaster* and *Geodia barretti*. In various places

of the North Sea young specimens of *Geodia eoaster* of 1—2 mm in diameter can be found which neither originated sexually nor through superficial buds. It is probable that they form regenerative cell-masses similar to the experiments dissociating the body of sponges. Burton investigated 100 specimens of the arctic *Geodia barretti* taken from the same bed, which were small sponges of an irregular shape 1.5—8 mm in length (Fig. 6 *A*).

As a result of histological investigations the author presumes that these sponges developed from reproductive body-constituents similar to that observed in the experiments of squeezing the sponges. Burton squeezed sponges through a silk cloth. The turbid fluid so obtained was added in drops to pure sea-water in a container at the bottom of which numerous cell-groups formed resembling "reproductive bodies" in their superficial pseudopodium-like prolongations and other features (Fig. 6 *B*). The author supposes that these young *Geodia* specimens observed, have been formed "by a natural production of regenerating cell masses", *i.e.* a new reproduction mode of sponges.

According to recent investigations of Korotkova [18] the phenomena of true regeneration can be observed on various sponge species; nevertheless, somatic embryogenesis should be regarded as dominant for sponges. Accordingly, the experiments of Wilson, Müller, etc. must be re-interpreted. As is known, Wilson [34] in his experiments on the marine sponge *Microciona prolifera*, Müller [24] on *Spongilla lacustris* and *Ephydatia mülleri*, and other workers have shown that the body of sponges can be dissociated (*e.g.* by squeezing through a fine-meshed cloth) into cells and aggregations of cells. These experiments, having been sensational at that time, should now be interpreted in a new sense, meaning that sponges do not regenerate better than animals having a more complex organization, but by the disintegration of sponges, phenomena of somatic embryogenesis can be induced.

Probably certain sponge species have no capacity for true regeneration or only to a lesser extent. The experiments concerning the dissociation of the sponges can by no means be discussed as a demonstration of the regenerative capacity of sponges. During these processes, a large part of the cell-material will be destroyed, consequently, we cannot speak of the reconstruction of something pre-existent from a pre-existing "constructing material". In our opinion, phenomena of somatic embryogenesis closely related to that of asexual reproduction are very characteristic of sponges. It is to be regretted that up to the present there have not been satisfactory investigations regarding the early stages of embryogenesis of sponges. Nevertheless, our knowledge permits the conjecture that the beginning stages of sponge development in the course of the processes of somatic embryogenesis, provoked experimentally, are very similar to that noted of sponges developed from gemmulae. When squeezing sponges through a fine-meshed cloth, isolated aggregations of cells can be observed in the sediment, and in addition to this, an intense phagocytosis removing the dead cells can be seen. The development of the sponges starts from the remaining cell-aggregates.

Korotkova [17] performed experiments mainly on *Leucosolenia complicata*. She inflicted various types of injuries by using punctured burns. Figure 7 shows a case of somatic embryogenesis provoked by burning and injuring the sponges.

The experiments on hydras also demonstrate the main differences between phenomena of somatic embryogenesis and true regeneration. In our laboratory, Aizupet [1] squeezed specimens of *Hydra oligactis* through a silk

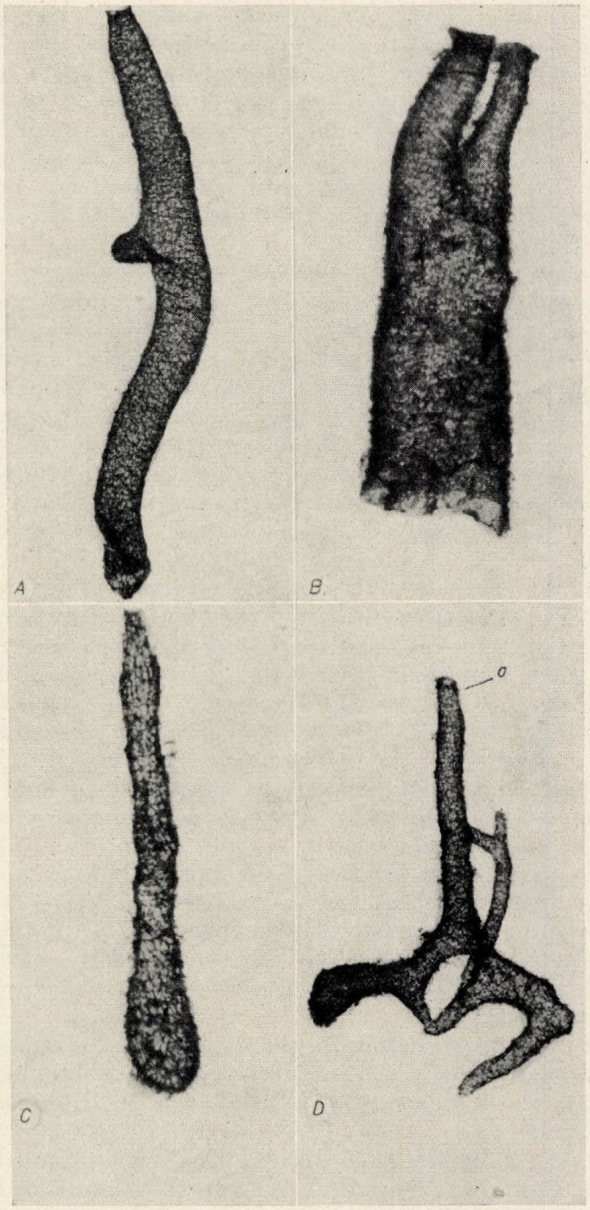

Fig. 7. Regenerating processes in the sponge *Leucosolenia complicata* provoked by various injuries. *A* — budding at the place of burning (15 days after operation, × 16); *B* — sponge with doubled apical pole (15 days after operation, × 16); *C* — sponge developed from isolated fragment of about 2 mm size (17 days after operation × 30); *D* — a sponge colony formed from some fragments under conditions of cultivation (20 days after operation, × 16); [17]

cloth and stated that, though the overwhelming majority of cells perish, certain fragments of 0.1—0.28 mm size will, nevertheless, develop into hydras. The interesting phenomena of cytotactic movement merely explain the formation of cell-aggregates or, at most, the development of ectodermal and entodermal cell-layers, but they cannot explain the re-establishment of the pre-existing hydra with its mesoglea and all its specific structures. No re-establishment of the pre-existing hydra takes place here, but a new organism develops by processes very similar to those occurring during asexual and sexual reproduction.

The view that phenomena of somatic embryogenesis are connected with the disintegration of cellular systems and the liberation of cells "from the prison of normal correlations" enduced us to experimentally provoke the development of hydras from gastral body areas lying outside the zone of budding. In our laboratory Tepliakova [29] applied punctured burns at suitable areas of *Hydra oligactis*. Similar results were obtained, at the same time and quite independently, by Strelin [27] who approached the problem from another angle. The details of Tepliakova [29] are of great importance: a considerable percentage of positive results can be obtained if the integrity of the hydra had been destroyed before burning, *e.g.* by cutting off its stem or its hypostome with the tentacles.

Certain experiments with the stalks of hydras demonstrate somatic embryogenesis [30]. After having strongly disintegrated the cell-systems of the stalk *(e.g.* by uniting the stalks and by repeated injuries inflicted on the stalk with a needle, then centrifuging the stalks, or by exposing them to sudden temperature changes at suitable intervals), the development of new hydras from the cells of the stalk of *Hydra oligactis* can be observed. Prior to the investigations of Trembley, this phenomenon was regarded as impossible. The success of the experiment depends on the disintegration of cell-systems within the stalk. It is obvious that in this experiment a re-establishment of lost parts cannot be expected.

Let us squash the gastral area of several hydras *(H. oligactis)* so that the maximal size of the fragments do not exceed one hundredth of the hydra's body. After all fragments have been mixed they are put into water containing glass-wool. On the second and third day certain fragments, visible to the naked eye, proved — when observed through a microscope — to be miniature hydras having tentacles and stalk.

Let us cut out a large part from the gastral area of *Hydra oligactis* and, after sectioning longitudinally the ring-like body-part, obtained sheet is fixed with pins to wax. These experiments also often show a tendency for a mass formation of hydras.

As shown by Zavarzin and Strelin [37] X-rays applied in a suitable dose inhibit the regenerative process, *i.e.* the restoration of lost parts *(e.g.* the restoration of parts of the hypostome with tentacles or that of the foot). According to my suggestion Babotshkina [2] controlled these data and supported them, showing, however, that the same X-ray doses inhibiting regeneration do not hinder the development of whole organisms from fragments of irradiated hydras. For example, a dose of 10,000 r resulted in an almost total inhibition of regeneration of the foot and the tentacles, whilst the development of whole hydras from ring-shaped parts cut out from the gastric region exceeded that of the controls *i.e.* the development of hydras from unirradiated

fragments (Fig. 8 *A*). These experiments clearly demonstrate that it would be misleading to unite in a single term the phenomena of regeneration and of somatic embryogenesis, fundamentally differing from each other.

In other experiments Babotshkina [2] kept ring-shaped fragments of hydras cut out from their gastral region in sterile salt solution (NaCl — 0.1%, KCL — 0.001%, $CaCl_2$ — 0.001%, $MgSO_4$ — 0.001%, $NaHCO_3$ — 0.002%) and starved them for several days. As a result two hydras instead of one developed from a single fragment (Fig. 8 B). Hydras injured with a needle were put into a sterile artificial medium and starved. As a result the tentacles were resorbed and the whole body transformed into a small amorphous corpuscle.

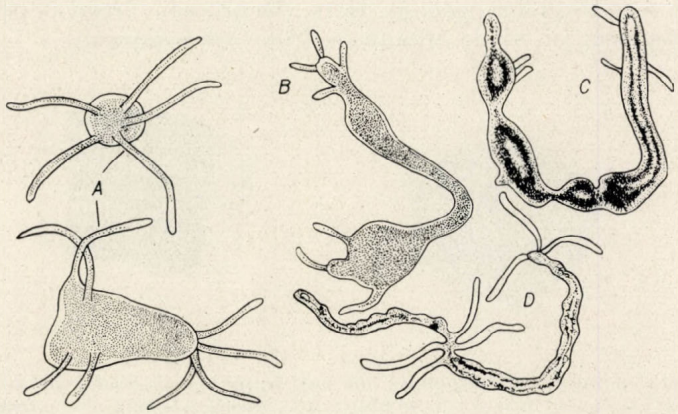

Fig. 8. Cases of somatic embryogenesis in *Hydra oligactis*. *A* — development of hydras from ring-shaped parts of the stomach region; dose of irradiation 10,000 r; *B* — development of hydras from similar fragments as in *A* under conditions of a sterile medium; duration of the cultivation was 8 days; *C* — hydra kept 10 days in sterile medium; *D* — the same hydra 3 days after having been replaced in a medium containing bacteria and infusoria [2]

Later Babotshkina put these hydras into water containing bacteria, protozoa and plants and observed that several deformed hydra-individuals were able to develop from a single hydra (Fig. 8 *C, D*).

It is doubtless that hydras have a regenerative capacity *i.e.* a capacity for restoring the lost body parts, but the capacity for somatic embryogenesis dominating in the hydra can either inhibit or strongly modify the processes of regeneration. The *Hydra oligactis* is undoubtedly capable of regenerating its pedal disk. Considering that the process of regeneration consists in the typical morphogenesis of certain parts of the organism and that it is determined by the remaining part of the organ or of other systems, it is evident that the pedal disk will regenerate only if the stalk, *i.e.* the part of the system which is in close contact with the lost part, is present. When the hydra is cut across the middle of the gastral region *(Hydra oligactis)* the pedal disk will not regenerate [15]. Hydras regenerate their tentacles cut off from the body with a small part of the hypostome, as well as each tentacle cut directly under its basis. However, it must be mentioned that malformations can be observed frequently *(e.g.* the tentacles will not be restored in their original number, etc.).

It is doubtful whether wound healing exists in hydras. Till recently, I did not doubt that hydras regenerate after having been cut at the middle of the gastral region and sectioned longitudinally, or cut into parallel cross-sections. This problem proved, however, to be extremely complicated. It is no mere chance that everybody having investigated the regeneration of hydras has observed partial malformations, heteromorphoses, the development of tentacles in unusual places and the formation of hydras with several head-poles. The smaller the fragments cut out from the gastral region the more frequently the phenomena mentioned above can be observed. Experiences of the last years have convinced me that frequent heteromorphoses and other similar malformations must be regarded as evidence showing the great capacity of hydras for somatic embryogenesis. Here, the process of regeneration switches to the development of whole organisms from cell-complexes.

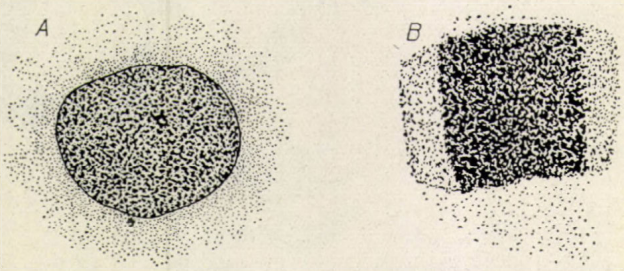

Fig. 9. Gradient of injury of fragments cut out from the gastral region of *Hydra oligactis* (in a 0.01% solution of methylene blue). *A* — decomposition of the fragment placed into the solution 16 hours after the operation; *B* — decomposition of the fragment placed into the solution immediately after isolation [2]

As mentioned before, somatic embryogenesis is preceded by a profound transformation of the cell-systems. This is clearly shown by the experiments of Babotshkina [2] and others, concerning gradients of destruction in hydras. A ring-shaped part cut off from the gastral region is immediately placed into a solution of methylene blue of a 0.01% concentration. An antero-posterior gradient appears: the gradual destruction of the fragment starts at its oral end (Fig. 9 B). When placing the same fragment into the same solution 16 hours or more after the operation, decomposition begins synchronously on all sides (Fig. 9 A). This fact shows that the fragment has become profoundly transformed, its organization and polarity have been destroyed. The antero-posterior gradient will show again when the development of the hydra begins and the tentacles start to develop. All this can well be supported by of histological investigations.

In cooperation with Bistrov, the author studied the development of *Hydra oligactis* from ring-shaped fragments of the gastral region by time-lapse microkinematography. Let us observe three moments of that process chosen at random. As shown by the film, the cell-material of the spherical fragment performs in the first hours of morphogenesis, very intensive movements demonstrating the reorganization of the cell-system. It is interesting that necrotizing cell material will be pushed out from time to time (Fig. 10 A).It is possible that this phenomenon is connected with the intensive katabolic

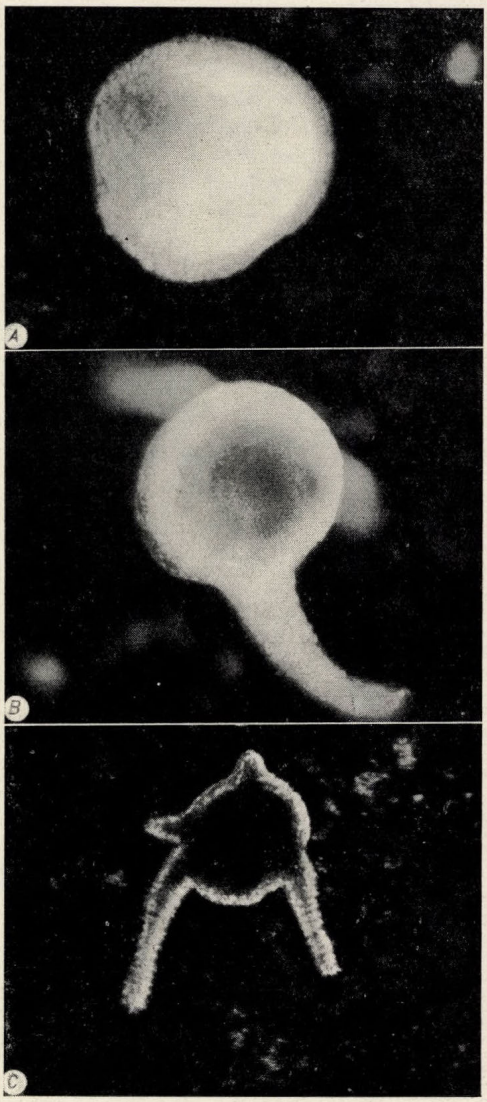

Fig. 10. Development of hydra from a fragment of the stomach region of *Hydra oligactis* (photo from a microfilm). *A* — elimination of the necrotizing cells (4 hours and 9 minutes after beginning the experiment); *B* — tentacles are forming on the opposite sides (24 hours and 52 minutes after beginning the experiment); *C* — all four tentacles are directed towards different sides (29 hours and 11 minutes after beginning the experiment) [orig.]

processes delivering energy for the fast morphogenetic processes. Finally, a very peculiar process can be observed: a certain trial of forming tentacles, at various points protrusions appear which then begin to elongate, however, most of them disappear later and only certain anlagen will develop into permanent tentacles. How do the stabilized tentacles develop then? It seems

that they will be formed quite asymmetrically as shown in the micrographs (Fig. 10 *B*, *C*).

Let us further mention the cases where whole hydras develop from body parts which under certain conditions have no capacity for regeneration. If the stalk of *Hydra oligactis* is cut at the region of budding, it will not regenerate and one or more new hydras will develop at the site of cutting (Fig. 11 *A*, *B*); sometimes, numerous hydras will be formed at this sites (Fig. 11 *C*). Hydras

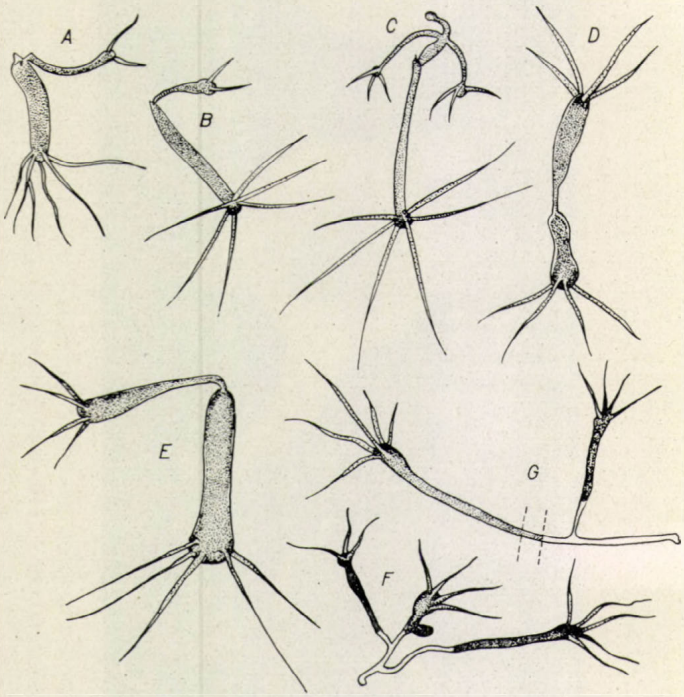

Fig. 11. Formation of new hydras from *Hydra oligactis*. *A*, *B*, *C* — 48 hours after cutting the stem at the level of budding; *D*, *E* — 48 hours after cutting the hydra into longitudinal halves; *F*, *G* — formation of hydras on the stems 3 hours after the same operation; region of the budding zone is dotted [orig.]

longitudinally cut to pieces very often show a process of somatic embryogenesis instead of regeneration. Instead of the regeneration of lost parts, the development of two or three new hydras will begin. In the course of this process, the stalk will atrophy (Fig. 11 *D*, *E*). We have observed the extraordinary case of new hydras developing from the stalk of hydras cut into longitudinal halves (Fig. 11 *F*, *G*).

These investigations convinced us that phenomena united under the single term: regeneration must be delimited from each other and that somatic embryogenesis is regularly preceded by the disintegration of the cell-systems. This regularity is valid for all organisms capable of developing whole organisms from somatic cells.

Till now embryology did not deal satisfactorily with the developmental processes occurring in the course of asexual reproduction. Nevertheless, the known literary data support the assumption that asexual reproduction is always accompanied by the disruption of the normal organization of organisms. This fact is well demonstrated by the development of statoblasts in Bryozoa or of gemmules in sponges. In our opinion, disintegrating processes occur also in that body-region of hydras which develops buds. It might be supposed that the various forms of asexual reproduction of worms involve similar phenomena *(e.g.* the strobilation, the architomy, the paratomy, the budding, etc.).

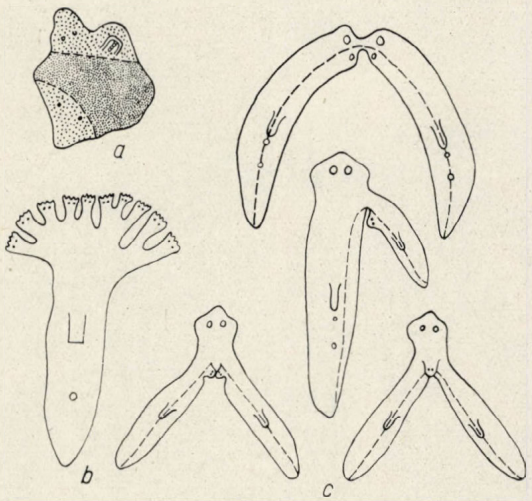

Fig. 12. Somatic embryogenesis in planarians. *a* — doubled monsters developed from small fragments of *Planaria lugubris* (as well as of *Pl. maculata* and *Pl. gonocephala) ; b* — hypermorphoses of planarians provoked by repeated incisions of the head region [21]; *c* — malformations in *Pl. lugubris* developed after different types of operations [19]

The groups representing a more primitive organization level, primarily flatworms, are specially important in this respect.

It should be mentioned here that the evaluation of experimental data concerning worms was made more difficult by the fact that the phenomena of regeneration and of somatic embryogenesis were placed in a single category. For example, the experiments performed on planarians often result in the formation of heteromorphoses, which must be regarded as products of incomplete somatic embryogenesis. We mentioned similar phenomena discussing the regeneration and somatic embryogenesis of hydras. As shown in Fig. 12*a* taken from the works of Lus [21, 22], two planarians are developing from a single body fragment so that a head and a tail pole form at the same time on the hind surface. Undoubtedly, this should be regarded as a process of somatic embryogenesis taking place in a malformed manner. Figure 12*b* shows the picture of a "planarian-monster" formed as a result of repeated mutilation of the anterior end of the body. In such hypermorphotic formations of the planarians it would be useless to look for any regeneration process.

The investigations of Davidov [9] performed in *Lineus lacteus* and other nemertinean species are of special importance for us. According to his data, fragments of the *Lineus lacteus* containing no intestine are capable of "regenerating" into quite normal organisms. The fragment cut out from the body will gradually become smaller whilst its tissues and organs differentiate; for example, its muscle cells will become similar to embryonal mesoderm-cells (Fig. 13); the cells in the wall of the blood vessels differentiate; the outer ectoderm reduces, the corium almost totally disappears, etc.

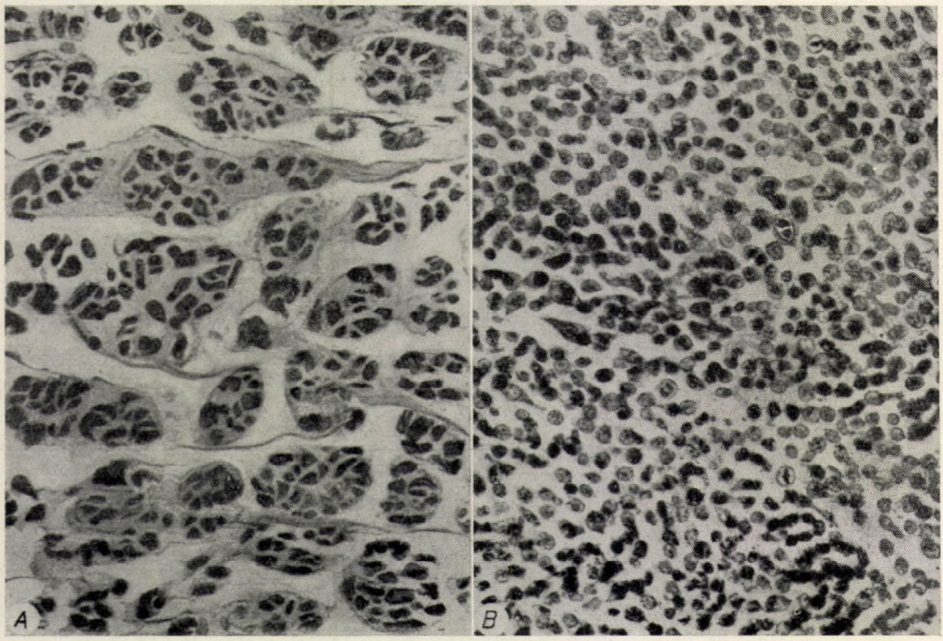

Fig. 13. Differentiation of the musculature in *Cerebratulus* after amputation. *A* — part of the normal musculature; *B* — part of the reduced longitudinal musculature [9]

Since Nemertini have a well developed capacity for somatic embryogenesis, their limited regenerative power can well be understood. Phenomena of true regeneration appear in various forms. For example, when the body of *Lineus lacteus* is cut into two cross-sections behind the cerebral organs, only the anterior fragment will regenerate, the hind one will regenerate no head.

As animal organization becomes more and more complicated, the capacity for asexual reproduction and somatic embryogenesis shows a gradual decrease. It is known that the echinoderms regenerate well enough, however, their somatic embryogenesis has not been studied satisfactorily. According to observations, the star-fish *Linckia multiflora* is capable to develop a new individual on the wound-surface on his arm. It is known that ascidians, especially their adult forms, having a less complex organization than their larvae, show the phenomena of somatic embryogenesis which were first described by

Driesch and termed as "regeneration" and "restitution". As to animals having a more complex organization, every case of somatic embryogenesis needs special analysis. In our monograph "Regeneration and somatic embryogenesis" [30] the problem is discussed in all its details. Here we must confine ourselves to a few examples.

It is a known fact that isolated blastomeras which can be regarded as somatic cells can develop into whole organisms. In these cases also somatic embryogenesis takes place. As shown by Holtfreter, the development of whole

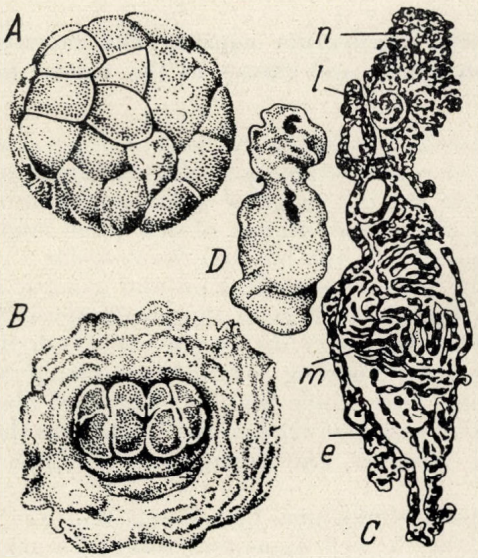

Fig. 14. Morphogenetic processes in isolated blastomeras of the triton at the stage of early blastula. *A* — early blastula of the triton; all cells, with the exception of the four marked ones, were killed with a needle; *B* — one hour after the operation, 8 cells are formed; *C* — embryo 9 days after operation; *D* — the same embryo in histological section; *l* — lens; *n* — nerve tissue; *m* — muscle segments; *e* — epidermis [Holtfreter]

organisms from isolated single cells can be provoked at the blastula-stage of amphibians (Fig. 14). Holtfreter killed the cells of the blastula with a few exceptions and observed that the cells left alive developed into malformed embryos containing tissue elements of all three germ layers.

The phenomenon of polyembryony can often be found in the animal kingdom and occurs in the group of the mammals too, *e.g.* in *Tatusia novemcinta*. Polyembryony can be regarded as a special case of asexual reproduction which has for its biological basis the disintegration of the developing germ at a certain stage of development.

In our monograph [30] we expound a hypothesis relating to the analysis of extremely interesting data delivered by the school of Spemann. I have tried to support the idea that the so-called induction of secondary embryos in developing amphibians, fishes, birds and mammals by using "living and killed organizers" is nothing else than a special case of experimental polyembryony.

It means that at certain developmental stages under conditions of the disintegration also vertebrates are capable of somatic embryogenesis.

When giving vent to the perilous scientific imagination, one might mention one type of the various human tumours, *i.e.* teratomas or, in other term, embryomas. Histological analysis can often demonstrate the presence of the derivatives of all three germ-layers within the teratomas. Teratomas can be regarded as a result of abnormal embryogenesis, closely related to somatic embryogenesis. Naturally, it still remains a very disputable problem.

Interrelations between regenerative capacity and somatic embryogenesis within the phyla and classes of the animal kingdom

The regular connection between the level of integration of the organisms and the phenomena of regenerations and somatic embryogenesis can be demonstrated not only in the full range of plant and of animal world but also within the different phyla and classes of the plant and animal kingdom. Let us choose for an example the phylum *Coelenterata*.

The capacity for asexual reproduction and somatic embryogenesis can be found in its most expressed form in the group of the fresh-water hydras as well as in cases of the marine *Hydrozoa* species. The hydras live 2—3 years reproducing themselves by budding, whilst their sexual reproduction occurs only 2—3 times during their whole life. As mentioned above, one can easily provoke somatic embryogenesis in the hydras. The hydras show also phenomena of true regeneration, however, the processes of somatic embryogenesis are dominating.

Tokin, Eritsheva and Kalinina [32] found that the hydrozoan species *Laomedea flexuosa* shows a well expressed form of somatic embryogenesis. The main statements are as follows.

Laomedea flexuosa (Fig. 15) has the capacity of regenerating its lost body-parts. For example, after having amputated the hydrorhiza of a colony showing normal physiological acitivity, it will be regenerated with its typical structure and normal functional characters (Fig. 16). As to the hydranths cut off from the colony, their regenerating phenomena are limited to processes which can be termed as "wound-healing". The isolated hydranths degenerate, however, in a short time. If the hydranths were cut without having been totally isolated, regeneration never can be observed and, in the first place, the lacking hypostome with tentacles does not regenerate. Regenerating processes of *Laomedea flexuosa* are very limited and stay behind that of the fresh-water hydras which, as mentioned, are characterized by dominating phenomena of somatic embryogenesis.

Whilst having a weak regenerative capacity, the *Laomedea flexuosa* is capable, in a very high degree, of somatic embryogenesis. This process can be provoked by cutting off the hydranths and by using punctured burns to injure the stolons and the foot of the hydranths. The development of new individuals (polyps) can occur also in the body parts of the *Laomedea* in which no asexual reproduction takes place under normal conditions (Figs 17 and 18).

The observations made on *Laomedea flexuosa* also support the hypothesis that somatic embryogenesis is preceded by the disintegration of cell-systems.

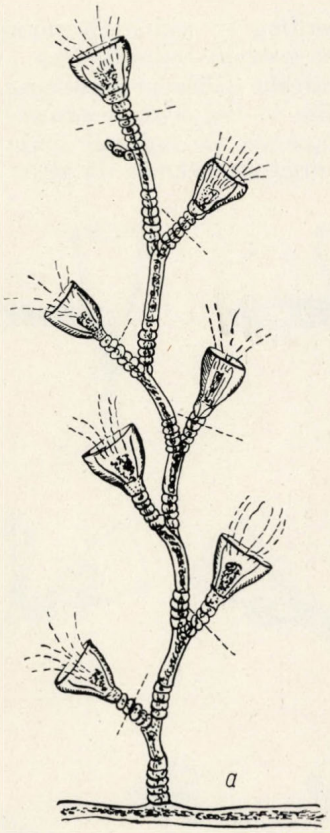

Fig. 15. Diagrammatic representation of the colony of *Laomedea flexuosa*. *a* — hydrorhiza (the cutting lines are dotted)

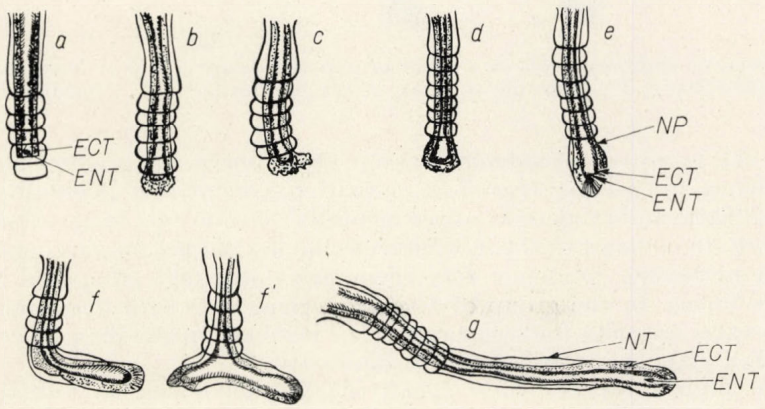

Fig. 16. Regeneration of the hydrorhiza of *Laomedea flexuosa* (scheme). *a* — hydrorhiza immediately after cutting; *b* — 12 hours after operation; *c* — 24 hours after operation; *d* — two days after operation; *e* — 3 days after operation; *f* — 4 days after operation; *g* — 5 days after operation; *ECT* — ectoderm; *ENT* — entoderm; *NP* — the new perisarc

The statement according to which the more primitive the forms the higher their capacity for asexual reproduction and somatic embryogenesis, can be demonstrated within the different classes *e.g.* within the class *Anthozoa*. The *Gastroblasta*, belonging to the hydromedusae of primitive organization, have a well developed capacity for somatic embryogenesis; the *Phyalidium* having a more complex organism shows no signs of the same phenomenon.

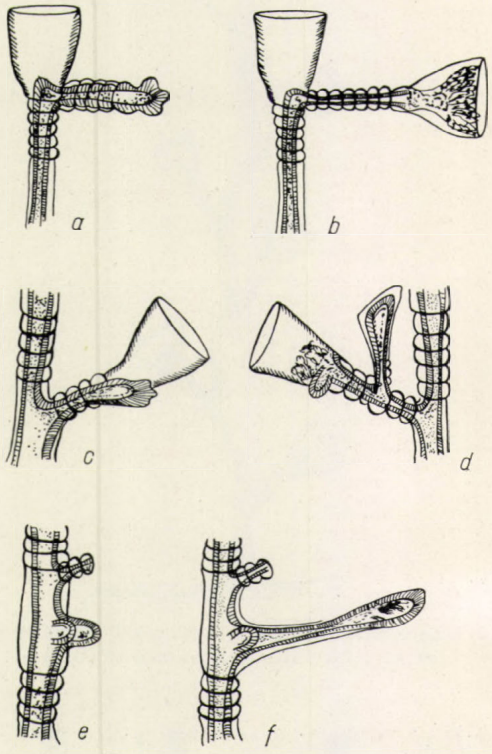

Fig. 17. Somatic embryogenesis as a result of punctured burns. *a*, *b* — burning the body of the hydranths; *c*, *d* — burning the foot of the hydranths; *e*, *f* — burning the stolon

In the group of *Coelenterata*, one can well observe the gradual decrease of the tendency for asexual reproduction (with the decreasing possibility of provoking somatic embryogenesis experimentally) within the range: *Hydrozoa* → *Ctenophora*. In contrast to this, *Coelenterata* having a more complex organization show an increasing tendency for regenerative capacity compared to more primitive forms. In the group of *Hydrozoa*, numerous hydromedusan species have a greater capacity for regenerating the lost body-parts than other species, *e.g.* hydras, having a more simple organization.

The known literary data speak about phenomena of wound healing and the replacement of lost parts in the medusae (for review see [19]). Regenerative capacity does not decrease in the group of *Scyphozoa*. The scyphistomas of the scyphomedusae have a well expressed capacity for regenerating lost parts (*e.g.* tentacles) and the regeneration process is often carried out during 1—2

days. The parts cut off from the radial lobes of the ephyra (*Mastigias papia*) will regenerate excellently. The manubrium also regenerates perfectly. Observations of Kaufman on planulae have delivered interesting data in details, too. He could observe no regeneration of parts cut off from the planulae, but he has found that planulae of the *Aurelia* or that of the *Cyanea capillata* show phenomena of somatic embryogenesis or other similar phenomena termed as

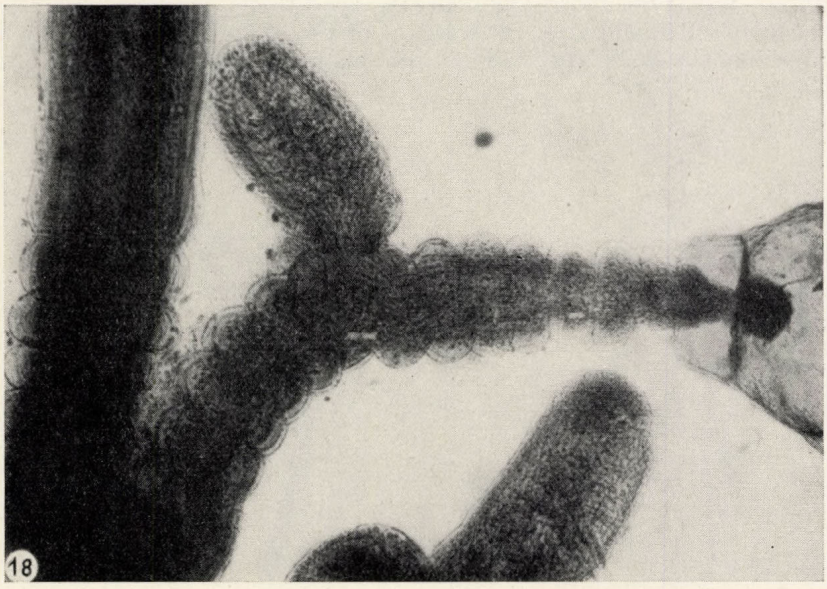

Fig. 18. Stolon-like formation at the region of the burn

regulative ones. The isolated halves of the planulae are capable of attaching themselves, it is possible that the planulae will coalesce with one another (Cyanea).

Anthozoa are generally capable of regenerating their lost body-parts to a great extent: they have the capacity for wound healing, for re-building various body-parts and organs, heteromorphoses can rarely be observed, etc. According to this, *Anthozoa* show phenomena of true regeneration and in a more expressed form than the more primitive Coelenterata, but phenomena of somatic embryogenesis are rare in Actinia. This process could be observed only in certain species staying on a lower level of organization. As an example the *Gonactinia* can be mentioned whose tentacles, when cut off from the body [25], are capable of developing into whole actinian organisms.

As to the highest Coelenterata, the *Ctenophora*, it was a general view for a long time, that these animals, according to their more complex organization, have only a weak regenerative capacity. However, as early as 1897 Eimer [11] observed the regeneration of the comb rows (ribs) in the Ctenophora. Mortensen [23] pointed out that the *Bolina infundibulum* is capable of regenerating its lost parts destroyed by burning. Coonfield [8] also has published data about the regenerative capacity of Ctenophora. In *Mnemiopsis leidyi*, he found

that the animals can regenerate (during 2—5—7 days) parts of their comb rows and the aboral organ. The author sectioned the body of ctenophore into parts and observed the regeneration of the body halves, quarters and eighths. Only in the last case could he observe no regeneration.

We investigated two ctenophoran species, *Beroe cucumis* and *Bolinopsis infundibulum* (31). The various operations performed are shown in Figs 19 and 20. In all experimental series we used at least 10 animals. The *B. infundibulum* was subjected to the following operations: (1) cutting off of whole comb rows or of their parts; (2) removal of the aboral pole; here, in one of the experimental series the section was made immediately in front of the

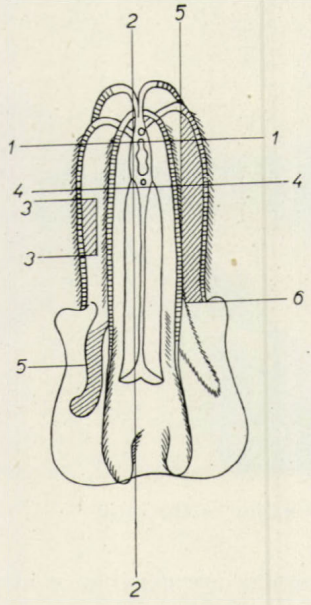

Fig. 19. Scheme of operations in *Bolinopsis infundibulum* [31]

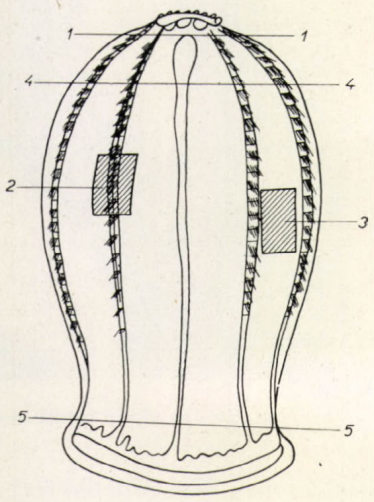

Fig. 20. Scheme of operations in *Beroe cucumis* [31]

stomach, whilst in other series the section plane went across the stomach; (3) the animals were longitudinally cut into two possibly equal halves but in one half, the aboral organ was missing; (4) we removed the auriculus, one of the lobes, etc. All these experiments have led us to the statement that *Bolinopsis infundibulum* has an excellent regenerative capacity. Total regeneration of the lobes undoubtedly takes place within 7 days, but it might only need 3 days. When the aboral end is cut off immediately in front of the stomach (Fig. 19, 1—1), this severe injury (removing the aboral organ, destroying the gastro-vascular system) will be followed within 7 days, by the re-building of all removed structures including the aboral organ. The removed aboral pole itself, containing the aboral organ, the endings of the canals and the stomach, develop into a whole organism within three days. The experiments including the longitudinal cutting of the animals are worth mentioning (Fig. 19, 2—2). After 17 days both halves of the body regenerated into whole organisms

(Fig. 21). On one of the regenerated body halves 8 rows of the ctenes could be seen (that is, the four lacking ones were regenerated). However, in place of the 16 ctenes the regenerated comb rows contained only 11 (in the short row of ctenes). In accordance with this, in the long rows of ctenes, the remained ones had 24 ctenes, but the regenerated rows had only 11. The regeneration of the comb rows begins from the aboral pole of the animal and is preceded by the regeneration of the meridional canal. The regenerated lobe was quite normal except its smaller size.

In the course of the operation not only the comb row was removed but also the meridional canal lying below it, as well as the corresponding part of

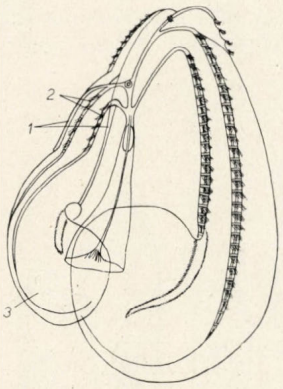

Fig. 21. Regeneration of removed body-half after longitudinal section of *Bolinopsis infundibulum* [31]

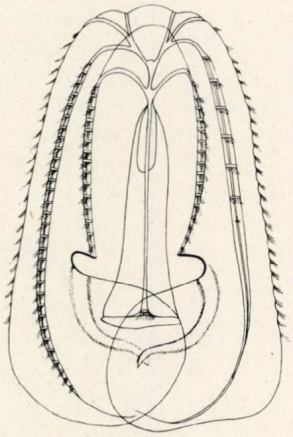

Fig. 22. Regeneration of ctenes in *Bolinopsis infundibulum* [31]

the nervous system; it was inevitable to remove a part of the mesogloea and the ectoderm between the neighbouring comb rows. It was all the more surprising that where the long comb rows were totally removed the developing new meridional canal could be seen after two days. The regeneration of the comb rows also started: in some specimens three of them were observed, in others four and in some five (Fig. 22). Within eight days, all operated animals had finished complete regeneration of the comb rows. We observed the regeneration of the auriculus, too.

As to the regeneration of *Beroe*, we only mention that the regenerative capacity could be demonstrated in all experiments (Fig. 20 shows the scheme of operation).

The facts mentioned above support the regenerative capacity of the ctenophores. In contrast to the view that the animals with a more complex organization necessarily regenerate to a lesser extent, it can be stated that in the range *Hydrozoa* → *Ctenophora* a progressive development of the regenerative capacity is seen. Our experiments to produce experimental somatic embryogenesis in ctenophores remained unsuccessful. The aberrant forms of ctenophores *(Coeloplana, e.g. Coeloplana bokii)* having the capacity for asexual reproduction should be mentioned too. The experiments of Okada [25] and

Tanaka [28] show that during the "regeneration" *i.e.* during the somatic embryogenesis of the *Coeloplana bokii* a profound reorganization of the "regenerating" parts of the body occurs.

Other animal types would deserve mention but recent biology does not present any satisfactory data. Nevertheless, certain comparisons could be made concerning worms, amphibians, and reptilians within the group of vertebrates.

Regeneration and ontogenesis

Our theory about regeneration and somatic embryogenesis includes the conclusion that ageing organisms show an inevitable decrease of regenerative capacity as a consequence of decreased function of their various integrating mechanisms. In senescence, the integration of tissues is changed; various

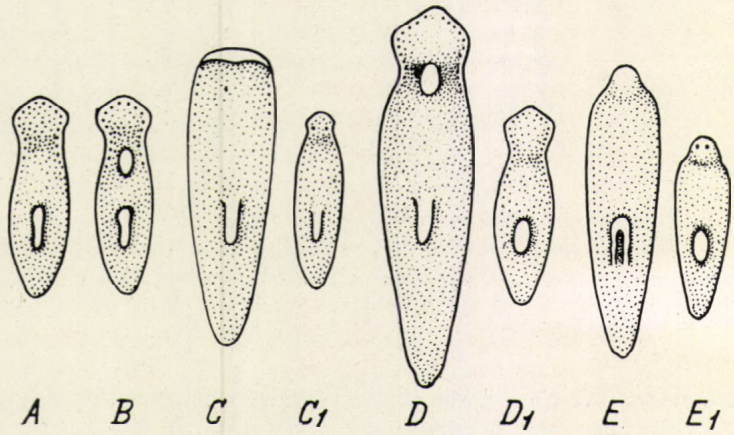

Fig. 23. Regeneration of one day old and of mature planarians after removal of the anterior part. *A* and *B*—level of cutting and site of burns in *Polycelis nigra; C* and C_1 — mature and young individual 4 days after operation; *D* and D_1 — the same 7 days after burning (light spot); *E* and E_1 — mature and young *Planaria torva* 7 days after operation (after Tsvetkova)

types of distrophy appear; phenomena of physiological regeneration, *e.g.* the processes of self-renewal of tissues, show a gradual weakening. All this must necessarily lead to the decrease and what is more, to the total cessation of normal regeneration as shown by investigations in human and animal surgery. Indeed, ageing organisms regenerate imperfectly, or they have no capacity at all for replacing their lost parts. This fact is well demonstrated by investigations made on amphibians and other organism (see [35]).

Many authors assert that young planarians regenerate to a lesser extent than old ones. This is not true, because the senescent planarians show phenomena of somatic embryogenesis rather than such of true regeneration.

Tokin and Tsvetkova performed investigation on *Polycelis nigra* and *Planaria torva* in order to clarify their regenerative capacity at different ages, *i.e.* at a one day old state and in the state of sexual maturity. The animals were decapitated as shown in Fig. 23 *A* or, in other experiments, they were injured by burning with a hot needle (Fig. 23 *B*). More than 300 successful operations have been performed. Young animals regenerated quicker than older ones. For example, the eyes appeared in the regeneration blastema of young animals on the 4th day, and in that of the adults on the 7th day. The young animal's head fully regenerated on about the 14th day (after decapitation),

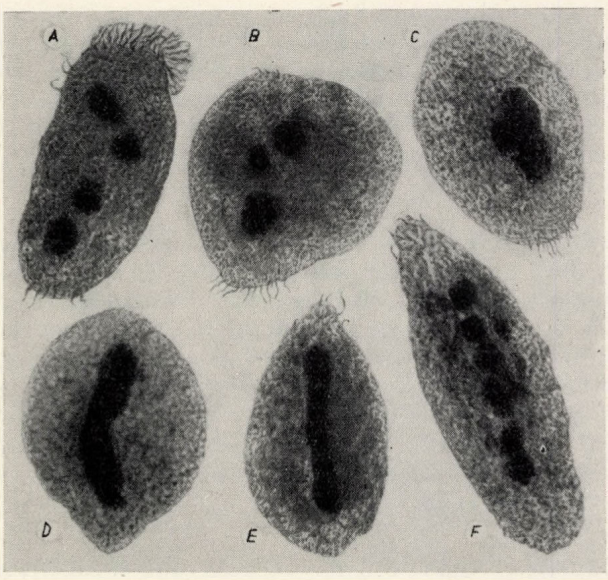

Fig. 24. Various effects obtained after the same cutting in *Onychodromus grandis*. *A* — animal operated at the age of 20 min., 4 hours after cutting; *B* — animal cut at the age of 8 hours, 30 min. after cutting; *C* to *F* — the same as in Fig. *B*, but 2, 3, 4 and 5 hours later [16]

whilst that of the adult animals took about a month. The experiments where injury was inflicted by burning gave similar results.

Ascidians are also noteworthy. As mentioned, adult ascidians show phenomena of somatic embryogenesis rather than those of true regeneration. This is explained by the fact that the organism of the ascidian larva becomes more simple, less integrated in the course of metamorphosis.

The observations concerning the regeneration of Protozoa deserve special attention. Most Protozoa, Ciliata in particular, are organisms with a complex organization and a high level of integration. According to many investigators, certain ciliata, *e.g. Calloscolex*, have an organization as complicated as Rotatoria; they have intestine, pharynx, mouth, complicated ciliary apparatus, various contractile elements, complex macronucleus, inner skeletal structures, etc. As to the complexity of the organization and the level of integration, most Protozoa are undoubtedly at a higher level than sponges and lower

coelenterates. This elucidates the fact that unicellular organisms, as shown by numerous works of various authors, have a more or less developed regenerative capacity.

In our laboratory, Karelina [16] has supported the earlier data of Bauer and Granovskaja [3]. She performed operations on the ciliate *Onychodromus grandis* removing about $^1/_{10}$ of its body. In individuals aged 20 minutes (*i.e.* in ciliates just finishing division), the regeneration will take place 4 hours after operation: the reconstruction of the nuclear apparatus cannot be observed (Fig. 24 *A*).

The same operation performed on sister individuals led to different results when performed *e.g.* at the age of 8 hours. 30 minutes after the operation, the macronuclei start to swell (Fig. 24 *B*) and after 2 hours the fusion of macronucleus parts, left after the operation, proceeds (Fig. 24 *C*); this process is particularly conspicuous 3 hours after the operation (Fig. 24 *D*). In this stage no limits between the nuclei of the macronucleus can be seen. In the 4th hour after the operation the contours of the future nuclei begin to develop (Fig. 24 *E*) and, finally, in the 5th hour the reconstruction of the nuclei will be completed.

Consequently, the removal of parts of the senescent organism provokes no regeneration, but it starts reorganizing processes within the injured body the biological importance of which is analogous with that occurring prior to the division. The operation of young individuals as well as the regeneration provoked by it do not modify the movement of the following division; on the contrary, the same procedure and the process of reorganization provoked by it act in the older individuals in a different way *i.e.*, the following division will be postponed by an interval between two divisions. As is known, in similar experiments Hartmann, by inhibiting the division of amoebas and the *Stentor*, has multiplied their life span.

Evolution of somatic embryogenesis and regeneration

The capacity for redeveloping lost body parts can be found in all organisms to a certain extent. This statement is supported by the mere fact that the metabolism of the protoplasm includes the ceaseless decomposition and re-building of its components. These processes constitute the main point of regeneration on the ultrastructural level of the cells.

The comparison of the regenerative capacity of different animals is very difficult. First it is very difficult to define the criteria of comparison. It is not sufficient, *e.g.* to consider merely the speed of regeneration processes. The regenerating capacity shows variations within the same systematic group and between closely related species, too. Among worms *e.g.* much *Rotatoria* and *Nematoda* regenerate to a much lesser extent than do *Tricladida* from the class of Turbellaria; the latter can be grouped differently according to their varying regenerative capacity, etc.

What then may serve as a basis for comparison of the regenerative capacity of various animals? According to certain authors, the regenerative capacity should be measured by the size of the lost and newly formed body parts. This assertion, however, cannot be sustained and originates, as mentioned already, from mistaking phenomena of true regeneration for those of somatic embryogenesis. The objective basis of comparison between the processes of

true regeneration is not easy either. The organization, the morphological and physiological features of recent representatives of the animal kingdom are specialized to such an extent that a comparison of their regenerative capacity seems, in certain cases, to be forced. For example, there is no real ground to compare the regeneration of the hypostome of the hydras and the bill of the birds. Only the comparison of the regeneration of homologous and analogous organs is justified, but in such cases difficulties can also arise in deciding which of the compared animals regenerates to a greater or lesser extent. It is extremely difficult to compare the regeneration phenomena of invertebrates with those of vertebrates, as well as to comparatively analyse phenomena of the reparative regeneration within the group of invertebrates, even if the group of Protozoa are not considered. There exists no common plan of

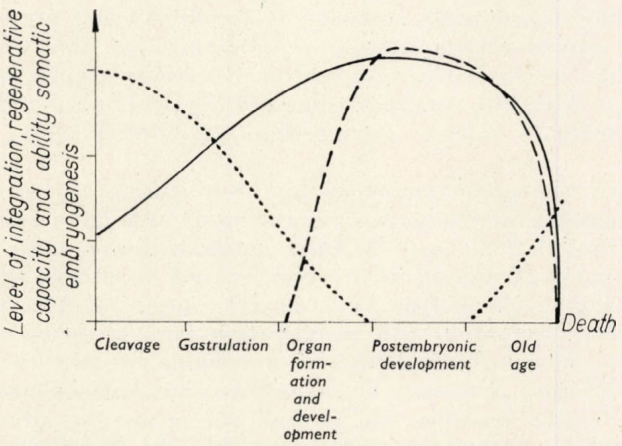

Fig. 25. Basic tendencies in the ontogenesis of animals of a high level of integration (scheme). Continuous line shows the changes of integration level during ontogenesis; dotted line the capacity to show somatic embryogenesis, and the interrupted line the regeneration power

organization in the group of invertebrates [4] and there are no straight developmental lines in the evolution of homologous organs.

In the course of evolution regenerative capacity altered not independently from, but in close correlation with other features, as *e.g.* asexual and sexual reproduction, the immuno-biological features, the life span, etc., can similarly be of great importance as to the progressive development of species.

Consequently the regeneration phenomena of animals can be compared concerning their qualitative rather than their quantitative features. This, however, does not mean that the degree of regenerative capacity *(e.g.* the speed of regenerating processes, the measure of replacing lost parts, etc.) would not be comparable within certain groups. Similarly, we can compare the regeneration of the homologous and analogous organs of the related animal groups.

This does not mean that a comparison concerning regenerative capacity is not possible when based on the integration level, however, caution is required.

As to the evolutive tendencies of reparative regeneration, the comparison of phenomena of physiological regeneration may furnish valuable data, and quantitative criteria can also be found. It is to be regretted that we have no

satisfactory data in this connection. For example, data about the intensity of "using up" and replacement of cells within the various organisms might also contribute to solving the problem to the extent the evolution of reparative regeneration was connected with that of physiological regeneration.

In connection with regenerative capacity, a frequently mentioned example is that the adult newts as well as the anuran larvae regenerate much better than the mammals. Moreover, this example is interpeted as demonstrating the gradual decrease of regenerative capacity in the course of evolution. It is true that mammals are not capable of regenerating their removed extremities. Nevertheless, this does not mean that all their tissues and organs regenerate to a lesser extent than those of amphibians. However, the same example, interpreted in the opposite sense, might lead to the conclusion that regenerative capacity does not decrease in the course of evolution, but, on the contrary, it increases. The regenerative capacity of amphibians by far exceeds that of several other groups with a simpler organization, *e.g.* that of the *Mollusca*. Such arguments based on the comparison of various animal species have no scientific value. After all, certain tissues and organs of man and the mammals do not regenerate to a lesser extent than the corresponding ones of lower organisms.

Which are the main tendencies in the evolution of processes of reproduction, regeneration and somatic embryogenesis? We have seen that all these phenomena differ in their genesis, their mechanism and their end result. In certain cases parts of organisms are regenerated, in others whole organisms develop from single cells or from isolated cell complexes. According to certain views, asexual reproduction and regeneration have a common origin as to their evolution. In our opinion this is improbable because these phenomena are based on different processes. For example, a low level of integration makes asexual reproduction possible, but it does not mean any advantage to true regeneration. These different processes can even be considered antagonistic. Asexual reproduction gradually lost importance and remained an important form of reproduction only in plants and in animals with a low level of integration.

The possibility cannot be excluded that certain forms of asexual reproduction might have developed secondarily in some animal groups. The polyembryony of higher animals is a good example.

Some form of regeneration phenomena can be found in all living organisms. They appear, however, in plants and in animals of lower organization in a less conspicuous form than in organisms of higher levels of organization. This statement is supported, *e.g.* by the excellent regenerative capacity of many vertebrates as shown by certain regeneration phenomena in birds, mammals and man.

All this is in marked contradiction with the well-known hypotheses of Pribram, Weissmann, etc.

The interrelations and the evolution of regeneration and reproduction phenomena suggest the following conclusions.

In accordance with other authors, we think that regenerative capacity is one of the most ancient properties of all organisms, since the metabolism of the protoplasm is accompanied by a ceaseless structural decomposition and reconstruction. Metabolism is not merely a complex of biochemical processes but it includes structural transformations, too, and therefore in the earliest living systems physiological regeneration already appeared in close connection

with metabolism. In the further course of evolution the various alterations of metabolism, organization and integration have naturally led to a further development of physiological regeneration phenomena.

Phenomena of reparative regeneration developed according to physiological regeneration and their further evolution proceeded in close connection with it. Processes of reparative regeneration connected with the self-renewal of cells and tissues became then more and more perfect and aquired special importance and features in the various representatives of the living world. By natural selection regenerative capacity has remained a useful biological feature and gradually improved depending on the special organization and the environmental conditions of organisms. Processes of regeneration developing in close connection with the integration of organisms have assumed different characters in the various organisms so that it would be forced to look for some common mechanism. Various processes of regeneration might be compared with one another, but they cannot be taken for identical processes.

Let us consider the problem of asexual reproduction and somatic embryogenesis. As mentioned, asexual reproduction and somatic embryogenesis are characteristic for organisms with a low degree of integration. Asexual reproduction of organisms with a more developed integration occurs only at simpler and less integrated physiological and developmental stages. The biological basis of somatic embryogenesis is, in all cases, the disintegration of tissue systems. Consequently, the evolution of reproduction and regeneration cannot be simultaneous. Both regeneration and asexual reproduction are probably very ancient properties of living organisms, but their evolution might have different causes.

Many zoologists, *e.g.* Hertwig think that asexual reproduction of the multicellular organisms may be regarded as a newly acquired characteristic developed in connection with special environment conditions. The appearance of asexual reproduction in multicellular organisms is presumably due to the fact that, as a result of evolution, cells which potentially represent all characters of the organism as a whole, have aquired the capacity to develop into whole organisms. As to sexual reproduction, we share the view accepted by the majority of biologists, that this form of reproduction originates from some type of asexual reproduction. It can be supposed, nevertheless, that sexuality represents an entirely new phenomenon which developed independently from asexual reproduction and whose similarity with the later consists in the capacity of certain cells to develop into whole organisms. The cell, as an integral part of multicellular organisms, potentially represents the whole organism. Thus, evolution has led to a dialectic contradiction given in the nature of multicellular organisms, and set forth lively discussions among embryologists, genetists and evolutionists.

In the present paper, we do not deal with the cytological and histological problems connected with regeneration and somatic embryogenesis. In our monograph [30] we attempted to give a new interpretation of differentiation and determination, further a comparison of somatic and germ cells. We developed our hypothesis of cellular ontogeny and demonstrated that cells undergo a partial differentiation during mitosis. Most somatic cells which did not lose their capacity to divide, can become analogous to the germ cells (if an adequate disintegration of the tissues or organisms takes place).

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Contributory Discussion

Prof. Faludi: A few years ago when the question of regeneration was discussed at a conference of the Academy, I expressed the view that the most classic form of regeneration was that of plants and called it symbolically a compensatory regeneration; while the other process under review is, strictly speaking, no regeneration but a general phenomenon of vegetative proliferation. I rejected the concept that regenerative power diminished in the course of evolution. I did not know Prof. Tokin's regeneration theory, and formulated my concepts somewhat inadequately, saying that the area of regenerative power became narrower with the progress of evolution but grew proportionately more efficient in the remaining area. I am glad that Prof. Tokin wishes this symposium to classify phenomena and to settle certain terminological problems. There is a question which, in spite of what has been set forth here, I still fail to see clearly, *i.e.* somatic embryogenesis. It undoubtedly belongs to regeneration phenomena, but I am not certain whether the term "somatic embryogenesis" is suitable to express the manifestations under review. I think this term is correct if used in connection with the frequent botanical phenomena in which embryogenesis originates in gametophytic or sporophytic cells. What, in my opinion, distinguishes embryogenesis from the various phenomena of neogenesis and neomorphism is that individuals arise there and not proles. Hence, ontogeny begins anew, with complete recapitulation, while an already existing process of ontogeny is continued in a changed or unchanged form in the other group. Cells which are close to or form part of the sex organs are not yet governed by the organism's integrative mechanism and are not yet specialized in embryony or apospory, *i.e.* those phenomena which I consider somatic embryogenesis in plants. All other phenomena arise from a cell or cells which are or have become more or less independent of the various regulative mechanisms of the integral organism. In the other case, discussed here, differentiated tissues already become dedifferentiated, and it is after the termination of existing correlations that embryogeny begins. The two processes are genetically quite different: the one represents neoformation (neogenesis) arising from an existing genetic basis, the other is true embryogeny based on a genetically new foundation.

Prof. Krompecher: I also have observed that a correlation of tissues facilitates regeneration. The formation of new articulations permits certain conclusions concerning wound healing and the process of regeneration in humans. If we detach the articular surfaces and leave them at rest, the contiguous bones will knit and scar tissue will form while, if adequate movement is ensured, fresh articular surfaces will develop. A correlation of tissues is, therefore, essential whether one is dealing with dogs or humans. I do not think this is essentially different from the processes occurring in orthogenesis, for the flexor and tensor synergism ensures a kinetic stimulation without which no joint can develop in the course of embryogenic growth. We have to ensure this stimulation if regeneration has to be accomplished at a later stage. Indeed, the theory that regenerative power diminishes with advancing phylogeny needs modification. It is not enough to consider the regenerating part alone: we have to consider histological correlations and functional facilities.

Prof. Kellner: I have the feeling that the lecture finished exactly at the point where it had become most interesting for us. My first question is: In which phase of phylogeny does somatic embryogeny cease to occur? What is the correct definition of somatic embryogeny? If *e.g.* only 15% of the liver remains, and a structurally complete new liver develops from this stump, can we speak of somatic embryogeny or are we faced with a radically different phenomenon? Further: what actually does integration, as referred to by the lecturer, mean? It is, according to my interpretation, a sort of help for the organism by a collective development of various kinds of cells, tissues, endogenous secretion and nervous apparatus. No clear definition has been advanced. How are integration and differentiation interconnected, a very significant point for regeneration? How are integration and differentiation correlated with senescence?

Dr. CSABA: As far as I have grasped the matter, the supposition is that regeneration arises from certain, let us say basic, cells that have survived. A new factor in somatic embryogeny is the process of dedifferentiation which gives rise to new structures. The problem, therefore, consists in whether and how far the two processes can be held apart, further, whether the concept of somatic embryogeny can be applied to processes of restitution in which the original matter is markedly dedifferentiated.

Prof. TOKIN: All questions raised here have been elucidated or at least discussed in my book "Regeneration and Somatic Embryogenesis". I suggest, therefore, to expatiate upon a central problem only, namely, how the process occurring in man should be interpreted from an evolutionary aspect. I have stated my opinion in the above monograph that the phenomenon of regeneration occurs in some degree in all organisms, since there is no organism without metabolism. Dissimilation is a biochemical and morpho-physiological process, and dissimilation occurring in the protoplasm is accompanied by destruction. I regard metabolism as the primeval form of regeneration; physiological regeneration in animals arose from metabolism. If we accept Darwin's principles and his theory of evolution, it is natural that Darwin's laws have to be applied to the phenomenon of physiological regeneration. This has also undergone evolution and reached that highest degree in man which we observe as physiological regeneration in the skin, in the intestinal tract, in the genito-urinary organs, etc. Historically, reparative regeneration arose from intensified physiological regeneration. Evolution shows a progress of both physiological and reparative regeneration. Evolution does not, of course, follow a straight line. Asexual propagation is also a primordial phenomenon of life which preceded sexuality. Both regeneration and asexual propagation are connected with metabolism, but, nevertheless, they are independent manifestations. Evolution shows two opposite trends: a tendency towards the decrease of asexual reproduction, and towards increased regeneration. Prof. Kellner raised the question in which phase of evolution somatic regeneration came to be arrested. Being a biologist, I cannot pretend to more than scientific conjectures in this respect. Let me refer to teratoma, a kind of neoplastic growth well known to oncologists. It is conceivable that teratomas are a specific, though distorted, manifestation of man's somatic embryogenic capacity. Teratomas are often regarded as misplaced embryonic structures with the appearance of all three germinal layers. I have collected and published in my work all available data concerning somatic embryogenesis, including polyembryony, *i.e.* the production of two or more embryos from a single egg.

Prof. ROMHÁNYI: I have heard with great interest that the regenerative power of protozoans depends on whether they are exposed to the critical action immediately after cell-division or only later. The metabolic process mentioned by Prof. Tokin which occurs in the postmitotic phase seems to ensure constant regeneration. Since life is macromolecularly organized, and since macromolecules are unstable, regeneration progresses continually. Is there no connection between the phenomenon under discussion and the fact that regeneration *i.e.* the redoubling of nucleic acids, occurs during the postmitotic interphase? The temporal interrelation of regeneration and metabolism might thus be studied.

Prof. TÖRŐ: I should like to learn something about the significance of quantitative factors: how does the number of cells influence these proliferative, regenerative and somatico-embryogenetic processes?

Prof. TOKIN: I am greatly impressed by Prof. Romhányi's contribution. Protozoans undoubtedly raise complicated problems; they undergo a great number of noteworthy changes between two divisions, and I think that, while studying the ontogeny of protozoans, we ought to examine the dynamics of nucleic acids, proteins and other biochemical phenomena between two cell divisions. It should, however, be understood that it would be wrong to view these phenomena only in their macromolecular aspects, and that it is not possible to study a number of phenomena merely at a cellular level. Not more than a few dozen works which contain accurate and reliable descriptions of asexual propagation are known to embryology. When speaking of phenomena of somatic embryogenesis which are so close to asexual reproduction, I purposely did not touch upon the morphological aspects of the matter, knowing that these processes may manifest themselves in many different forms. A study of plants and primitive multicellular animals reveals that evolution may start from single cells as well as from cell-groups. The number of cells plays no role in this respect: the only important problem is whether the original tissues survive or disintegrate. As for their chromosomal equipment, *i.e.* their possibilities, somatic cells do in no way differ from fertilized germ cells. Genetics make it clear that somatic and germ cells may be analogous. Embryologists have not concerned themselves with this problem, so that further investigations are necessary.

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ACTIVITY OF INTRACELLULAR ENZYMES IN REGENERATING NEWT LIMBS

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Synopsis

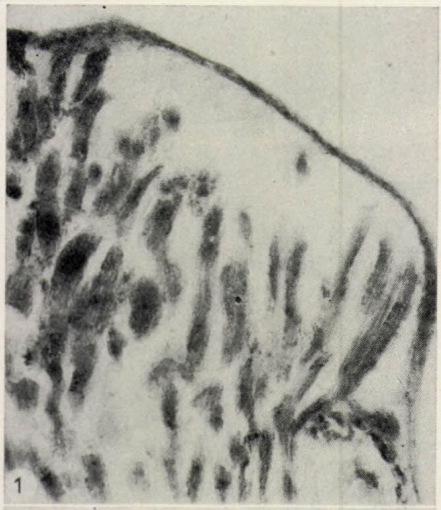
The activity of diphosphopyridine nucleotide diaphorase (DPND), triphosphopyridine nucleotide diaphorase (TPND), succinate dehydrogenase, cytochrome oxidase, glucosan phosphorylase and β-glucuronidase was histochemically investigated in regenerating newt forelimbs. An increased activity of DPND and cytochrome oxidase appeared in the wound epithelium about 24 hours after amputation, that of succinate dehydrogenase and TPND at the end of the first week. Wound epithelium cells were glucosan phosphorylase positive about 24 hours after amputation but normal epidermis on the stump did not react. Striated muscle fibres showed the highest activity of this enzyme. The activity of β-glucuronidase developed like that of oxidative enzymes.

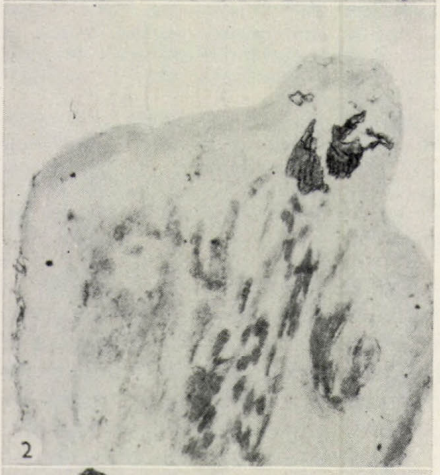
Dedifferentiation was marked by a general decrease of activity in dedifferentiating tissues: bone, cartilage and striated muscle. However, dedifferentiating osteocytes and striated muscle fragments showed moderate to rather intense activity. The reactivity of blastema cells was always inferior to that of the wound epithelium. During differentiation, normal enzymatic activity was gradually restored to the particular tissues of the regenerate.

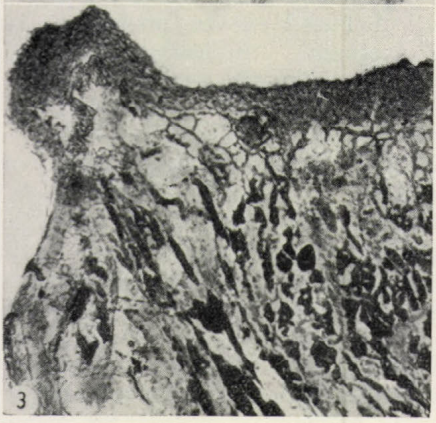
Since the eighteenth century, in which the modern investigation of regeneration phenomena in animals was started by Trembley, chiefly morphological events were studied in the process of regeneration. The progress of contemporaneous biochemistry and histochemistry gradually made it possible to look for a solution of developmental enigmas in regenerating organs by means of methods based on these sciences. According to modern views, regeneration through tissue regrowth should be regarded as the primary type of regeneration in vertebrates, whereas the type based on the presence of a blastema is secondary. The development of blastema — the essential element of regenerating extremity — is doubtless an evolutionary product of cytochemically changed cells and tissues of the wounded organ. Consequently it became necessary to investigate regeneration also cytochemically.

Some of the data presented in this paper were collected in the Department of Pathology of the Postgraduate Medical School in London, others in the Department of Experimental Zoology of the Polish Academy of Sciences in Krakow. The material used in this investigation consisted of seventy adult newts, both male and female, of the species *Triturus alpestris* and *Triturus vulgaris*. The animals were kept in water at 25° C. The forelimbs were amputated near the elbow. The developing regenerates were successively removed at short intervals during 4 weeks after amputation for histochemical investigations.

The regenerates of the species *vulgaris* were sectioned at 10μ in a remotely controlled cold microtome (cryostat) and the sections processed for histochemical localization of the diphosphopyridine nucleotide diaphorase (DPND),







Figs 1 to *3*. Regenerated limbs of (1) *Triturus alpestris*, 24 hours after amputation, β-glucuronidase, × 90, (2) *Triturus vulgaris*, 48 hours after amputation, cytochrome oxidase, ×38, and (3) *Triturus vulgaris*, 48 hours after amputation, DPND, ×48. The wound surface on the top is covered with epithelial cells reacting more intensely than those on the stump. The connective tissue in the wound region reacts weakly, the striated muscle intensely

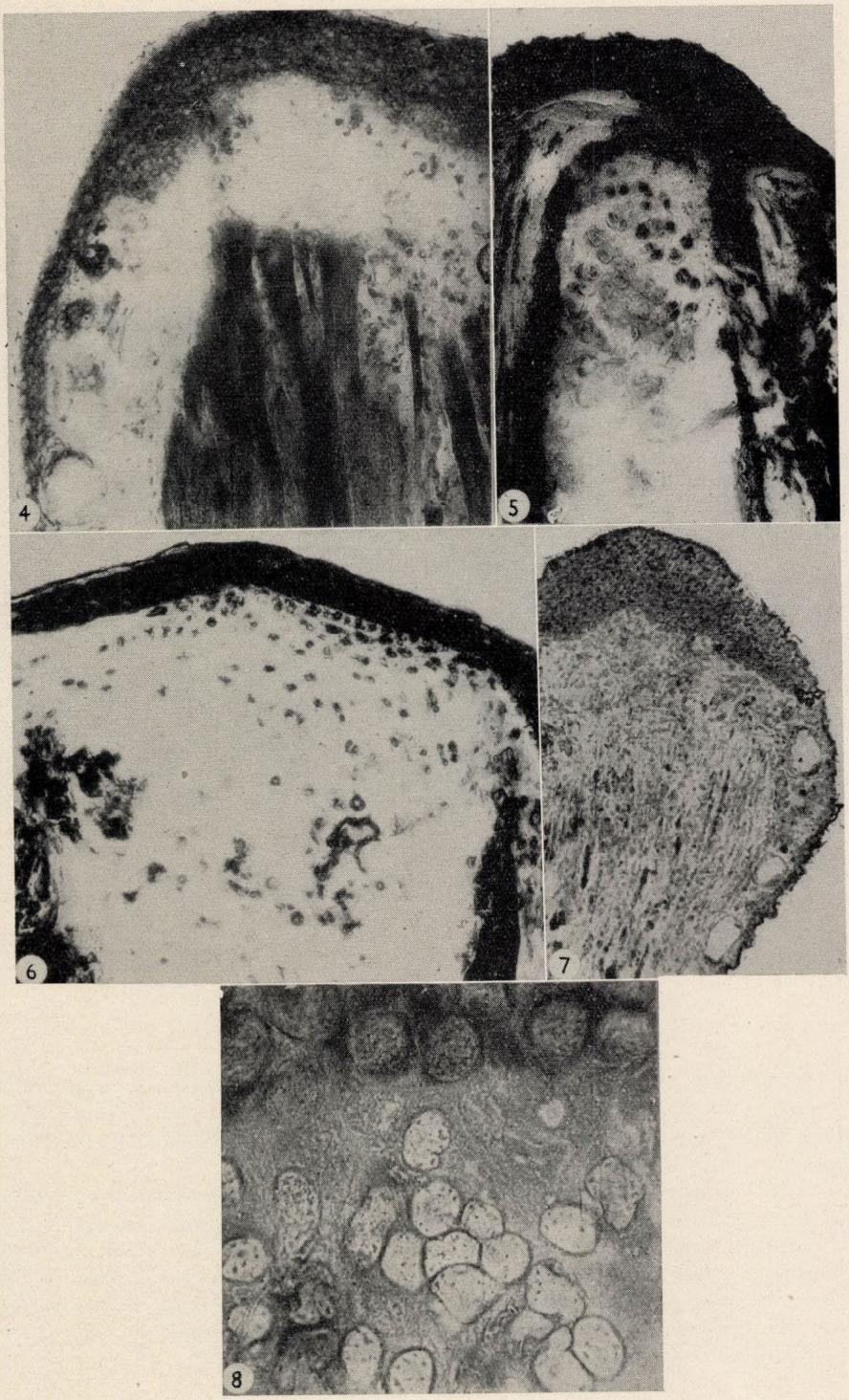
Fig. 4. Triturus alpestris, 48 hours after amputation, β-glucuronidase, ×90. The activity in the wound epithelium is strongly increased. The fibrocellular tissue is non-reactive

Fig. 5. Triturus alpestris, regenerating limb 4 days after amputation, β-glucuronidase, ×90. Under the strongly reactive epithelium dedifferentiation of the striated muscle proceeds proximal. The increased reactivity of the osteo cytes in the distal part of the dedifferentiating bone fades in the proximal direction. The periosteum reacts moderately

Fig. 6. Triturus alpestris, 5 days after amputation, β-glucuronidase, ×90. The first blastema cells appear among the connective tissue elements under the strongly reactive wound epithelium. Intensely reacting remnants of dedifferentiating skeletal muscle are seen under the blastema area

Fig. 7. Triturus vulgaris, 8 days after amputation, DPND, ×48. The cap formed by the intensely reactive wound epithelium is easily discernible from the stump epithelium which reacts moderately. The process of dedifferentiation is advanced far proximal and only small remnants of skeletal muscle react strongly. Under the epithelial cap groups of moderately reacting blastema cells are seen

Fig. 8. A detail of Fig. 7. Moderately reacting blastema cells are seen embedded in the connective tissue under the strongly reactive wound epithelium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triphosphopyridine nucleotide diaphorase (TPND) and succinate dehydrogenase according to the method of Pearse *et al.*, cytochrome oxidase (Burstone), and glucosan phosphorylase (Takeuchi).

From the regenerating limbs of the species *T. alpestris*, sections were cut at 25μ on a freezing microtome. They were subsequently examined for the distribution of the β-glucuronidase activity using the Fishman and Baker technique.

It was observed that a good activity of DPN-diaphorase and cytochrome oxidase appears in the cells of the epithelial wound cover within 24 hours

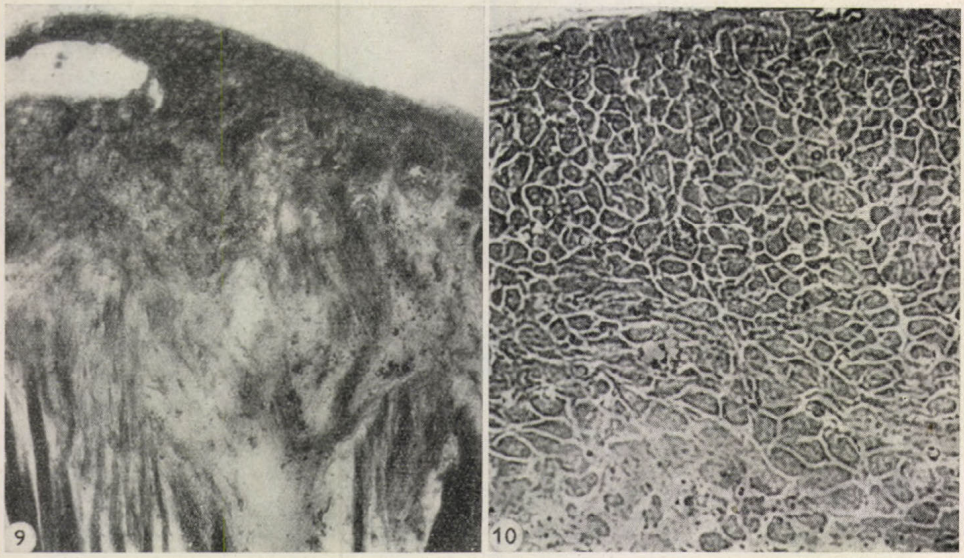

Fig. 9. Triturus alpestris, 10 days after amputation, β-glucuronidase, $\times$90. Under the intensely reacting epithelial cap formed by the wound epithelium groups of β-glucuronidase — positive blastema cells are visible. Non-dedifferentiated skeletal muscle fibres react strongly

Fig. 10. Triturus vulgaris, 10 days after amputation, succinate dehydrogenase, $\times$300. The epithelial cap formed on the wound surface is more reactive than the blastema cells at its lower margin

after amputation, the activity of TPND and succinate dehydrogenase at this time being much lower. At the same time these cells are glucosan phosphorylase positive which probably results from the utilization of carbohydrate in their cytoplasm, while they move onto the wound surface.

In the course of the following days the oxidative activity of the wound epithelium gradually increases and finally becomes equally strong for all the oxidative enzymes in question. Consequently, by the tenth day after amputation a sharp border is seen between the young wound epithelium and the old epidermis of the stump. The blastema cells appearing from the fifth day on are enzymatically less active than the wound epithelium cells. In the following days they increase their activity only slightly.

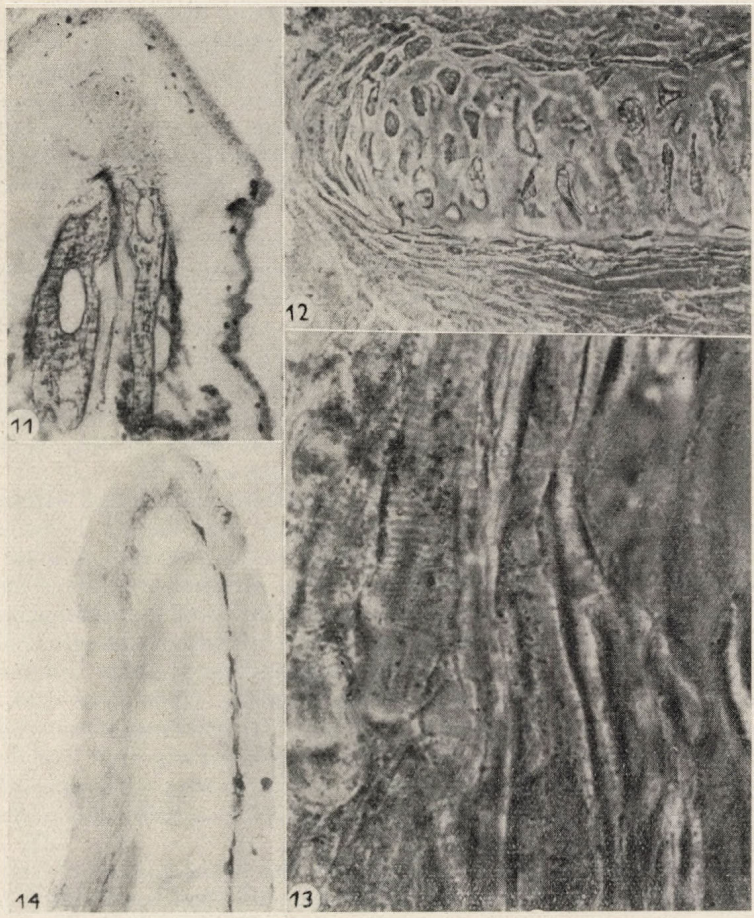

Fig. 11. Triturus vulgaris, 12 days after amputation, DPND, ×38. At the base of the regenerate, cartilage is beginning to form from reactive procartilaginous cells. The increased reactivity in the wound epithelium is no longer visible

Fig. 12. Triturus vulgaris, 17 days after amputation, DPND, ×300. The cells of the cartilaginous rod and of the perichondrium are more reactive than the surrounding undifferentiated cells. Near the rod dark groups of differentiating striated muscle anlagen are visible

Fig. 13. A detail of Fig. 12, ×1200. A group of differentiating muscle anlagen composed of elongate cells present a distinct striation. Groups of enzyme-positive mitochondria can be seen within the cells

Fig. 14. Triturus alpestris, 23 days after amputation, β-glucuronidase, ×90. The procartilage differentiating inside the regenerate forms a rod consisting of the moderately reacting chondrocytes around which a perichondrium develops. The cells of the latter show moderate to intense β-glucuronidase activity. The reactivity of the cells surrounding the tip of the cartilaginous rod is remarkable. Muscle anlagen developing in the proximal part of the regenerate react rather intensely

Meanwhile, histolysis of the dedifferentiating tissues of the stump proceeds proximal, so that only moderately reacting tissue remnants are visible on the amputation site. The onset of the process of differentiation by the tenth day changes enzymatic reactivity of the elements of the regenerating extremity. The activity of the wound epithelium decreases and equals that of the stump epidermis, while the blastema cells differentiating into chondrocytes of the cartilaginous rod, and subsequently also into striated muscle fibres, become distinctly more reactive [2].

The biological truth of the observed processes becomes convincing in view of analogical results obtained with the localization of β-glucuronidase in the regenerates. This enzyme, related to a sector of metabolism other than oxidation, has been found to be particularly active in cellular proliferation and in certain types of neoplasms. Unlike oxidative enzymes, the activity of β-glucuronidase in the blastema cells was relatively strong and only slightly inferior to the activity shown by the wound epithelium [1]. This phenomenon agrees well with the recognized role of β-glucuronidase in the organism and with the developmental potentiality of blastema cells [8].

It follows from the above data that dedifferentiation and differentiation represent two biochemically opposite processes, the former concerned with diminishing, the latter with increasing enzymatic activity. A good analogy to morphological events is obvious here. The lessened oxidative activity of blastema cells which represents the profound chemical metamorphosis, which is in progress in these cells, undoubtedly underlies their morphological simplicity.

The relatively good reactivity of differentiating tissue remnants agree with recent findings showing that skeletal muscle, fibroblasts and Schwann cells of the regenerating extremity contribute to blastema. Our study of the localization of β-glucuronidase revealed an increased reactivity of dedifferentiating osteocytes. However, with respect to the striking difference of activity between the epithelial and blastema cells, a direct contribution of the former to the blastema appears unlikely.

Similar biochemical data referring to the development of the activity of acetylcholine in *Triturus viridescens* were published by Singer [7]. It also appears that the gradual increase of the enzymatic activity in the regenerate might be compared with the constant increase of respiration in developing amphibian embryos. Similarly, both in embryological development and regeneration after the early rapid increase of the metabolic activity, a transitional period appears during which a slowing down of the tissue respiration occurs. The transitional period observed on the tenth day after amputation in the regenerates of different newt species and demonstrated by different histochemical methods, represents the outer effect of the intimate chemical changes taking place in the protoplasm. Its appearance coincides in time with the termination of the influence of the nerve fibres on the regenerating limb which, if deprived of nerves, cannot dedifferentiate [4, 5]. Withdrawal of nerves after dedifferentiation does not interfere with the subsequent growth and differentiation of the regenerate [6, 7]. The strong enzyme activity which precedes the transition marking the beginning of differentiation conforms to the views of the authors [3] who maintain that significant morphogenetic events, in this case the onset of differentiation, are accompanied by increased energy production. The real meaning of the transitional period which entails a relative decrease of the enzymatic activity in the regenerate and is paralleled by a

decline of respiration in amphibian and other embryos, is not yet sufficiently understood. There is, however, little doubt as to the correlation of these two facts with the metabolic shifts involved in differentiation.

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Contributory Discussion

Prof. Szodoray: Dr. Niwelinski, did you, or did you not assume that the regeneration processes depend chiefly on nervous stimuli, *i.e.* on the presence of acetylcholine?

Dr. Niwelinski: There is some evidence of the nervous control in the processes of differentiation. From the very beginning of regeneration nerve fibres are present in the epithelium covering the wound surface, they penetrate the tissues at the amputation surface reaching almost every cell in the regenerating part. Most likely, the nervous system exerts a chemical influence on the regenerate. A correlation is known to exist between the effect of acetylcholine and oxidative processes. The demonstration of oxidative enzymes and of a hydrolytic enzyme β-glucuronidase revealed similar results coinciding with the results concerning acetylcholine. The activity changes also show this distribution and the peaks coincide.

Dr. Török: Dr. Niwelinski presented data concerning intracellular enzymes as found in the normal extremities of newts, and the role these enzymes play in their regeneration. We observed a strong alkaline phosphatase reaction in the area of the neuromuscular synapses of planarians and attach great importance to it in connection with the conduction of impulses. We saw intensive alkaline phosphatase and cholinesterase activity in the entire central nervous system of *Dugesia lugubris*, an animal of marked regenerative capacity. There is practically no such activity in the *Dendrocoelum lacteum*, an animal which has scarcely any regenerative power, and in which even this weak regeneration is restricted to the anterior portion of the body. A correlation of this kind may be of importance, and it might be profitable to extend these studies to further species.

Dr. Niwelinski: Using Gomori's method Scotsman and Burns found in axolotls a high level of alkaline phosphatase in differentiating bones and adjacent tissues of the extremities for a few days after amputation. Subsequently also a high activity was found in differentiating bones and tissues of the regenerate. When differentiation was finished the activity was nearly normal. I never worked with planarians but I think they are worth while investigating because the data mentioned are quite different from those in vertebrates.

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ROLE OF NUCLEIC ACIDS IN THE WOUND HEALING PROCESS

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Synopsis

The author's cytochemical and biochemical investigations on nucleic acids in injured tissues have shown that the nucleoproteins are the injury-sensitive units of protoplasm. Mechanical injury, when irreversible, leads to a splitting of the ribonucleoprotein complex, followed by a rapid degradation of RNA. The liberated breakdown products proved to be the leucotactic substances set free by injured tissues. Reversible injury obtained by a properly determined mechanical pressure results in an activation of cellular multiplication preceded by a partial degradation of RNA and its subsequent resynthesis. It is very likely, therefore, that the reaction of nucleoproteins to mechanical injury is the starting point of regenerative processes after injury.

Introduction

It is well known that two essential processes are involved in the wound healing: demarcation of necrotic tissues by leucocytic emigration and restoration of tissue defects by cell proliferation. In both cases the injury of living cells appears to be the initiating mechanism of the following reactive processes. Reversible injury of cells, which is not connected with cell necrosis, activates only cellular regenerative processes, whereas irreversible injury results in a preliminary development of inflammatory demarcation processes. Evidently, in both cases some biochemical changes, which may be considered as an initial point of the wound process, take place at the moment when the cells are injured. In this connection it should be noted that according to previous investigators some unknown substances — the so-called woundhormones or necrohormones stimulating cell division — are liberated when the tissues are injured (for review see [66]).

Considering that the regenerative processes during wound healing are closely connected with protein biosynthesis, it is of great interest to discuss this problem on the basis of our present knowledge of the biological rôle of nucleic acids.

In the last ten years a number of works (for review see [11, 12]) have given strong evidence of the close relationship existing between nucleic acids and protein synthesis. In support of this idea one can also find the data of several authors showing an increased content of RNA during wound healing [4, 16, 28, 41, 53, 65]. This fact, however, being in accordance with the general view that RNA is indispensable for synthesis of new proteins, does not contribute to the elucidation of the starting mechanism of regenerative processes after injury. The importance of nucleic acids in synthetic processes, suggests

that they are the point affected by injuring agents. This is the reason why in a number of investigations made in our laboratory, special attention was paid to the alterations of nucleoproteins taking place at the moment of injury as well as in the following hours.

Nucleprotein alterations after tissue injury

Our cytochemical investigations [53] which were carried out on mouse skin according to the method of Brachet, have revealed a strong increase of the ribonuclease-sensitive protoplasmic basophilia appearing at the moment

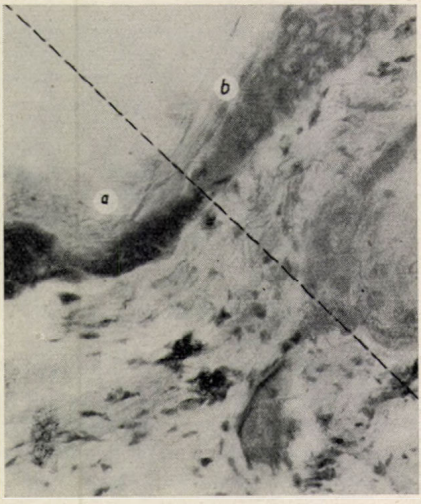

Fig. 1. Increased pyronin adsorption in mechanicaly injured cells (mouse skin). *a* — injured, *b* — intact area. Methyl green-pyronin [53]

of inflicting a mechanical injury (Fig. 1). An increased dye adsorption in injured tissues has been reported some time ago and according to the denaturation theory of Nassonov and Alexandrov [35] it is due to the liberation of active binding sites of the denatured proteins caused by injuring agents. Our investigations prove, however, that this explanation cannot be generally accepted.

The strongly increased pyroninophilia of mechanically injured cells is completely eliminated after ribonuclease treatment [53]. This directly proves that the increased pyronin adsorption after cell injury is to be attributed to RNA. Similar observations were made by Brachet, who reported that injured amphibian embryos exhibit an increased RNA basophilia [9, 10].

Trifonova [52] who also observed a heat-induced increase of pyronin binding in amphibian epidermic cells, pointed out that injury leads to an increased RNA content of the protoplasm.

However, our experiments have shown that the strong basophilia of injured cells is not due to an increased content of RNA. The increase of RNA

basophilia takes place at the moment of injuring the cells, this fact making a strong increase in the RNA content unlikely. Direct estimations of the nucleic acid content in mouse skin immediately after injury, reveal only a 10—15 per cent increase, the quotient RNA/DNA remaining unchanged [60]. This small increase in both RNA and DNA content is connected with some loss of soluble substances and cannot account for the increase of about 80% of pyronin binding (Fig. 2) [57].

According to our data the increased ribonuclease sensitive basophilia of injured cells is due not to quantitative changes in the RNA content but

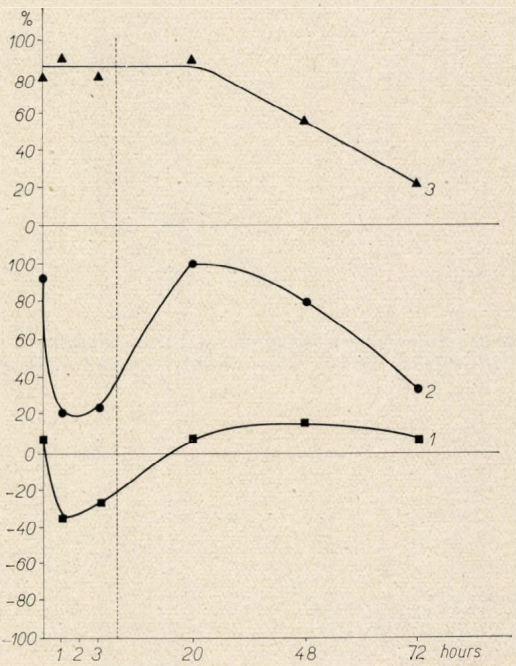

Fig. 2. Changes in the adsorption capacity of RNA in subcutaneous connective tissue (rat) after mechanical injury (7 kg/cm²). *1* — RNA content; *2* — Pyronin adsorption; *3* — Pyronin/RNA quotient. Abscissae — hours; ordinates — relative changes [57]

to altered relationship between RNA and proteins. As is known the basophilic adsorption sites of RNA are its phosphate groups. Blocking of these groups by linkage to proteins would result in the decreased capacity of RNA to bind basic dyes. Some data suggest that the phosphate groups of RNA in the protoplasmatic nucleoproteins are not free, since they are in strong interaction with different groups of the protein moiety [15, 48, 59]. On the basis of the pH dependence of pyronin adsorption we calculated that the apparent ionising constant of the phosphate groups of cell nucleoproteins is 100to 1000 times lower than that of the free RNA [59]. After cell injury this constant reaches the value of the free RNA, as can be seen by the dependence of staining on pH (Fig. 3) [15]. This proves that the phosphate groups of RNA are set

free by cell injury. In support of this statement is our finding that a more rapid hydrolytic liberation of phosphorus takes place in injured cells [53]. In connection with our data the observation of Larionov and Brumberg should also be mentioned [31]. These authors described injury-induced changes in the absorption spectrum of cell nucleoproteins which they attribute to the splitting of the bonds linking nucleic acids and proteins together. Summarizing all these data we have reason to assume that cell injury results in a dissociation of the

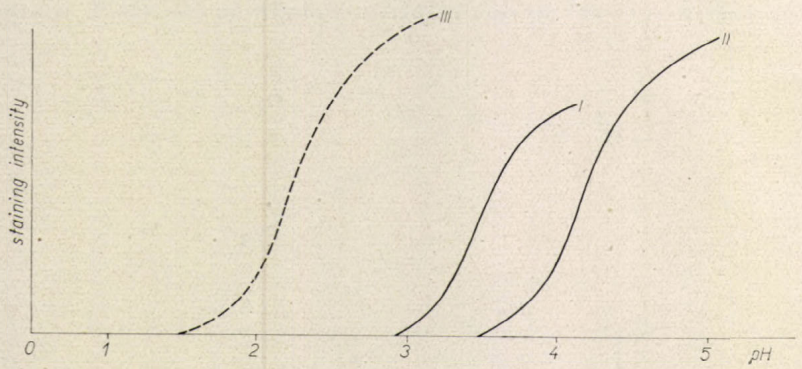

Fig. 3. Dependence of the adsorption capacity of RNA on pH. *I* — Normal epidermis; *II* — Normal fibroblasts; *III* — Irreversibly injured cells (rat tissues) [15]

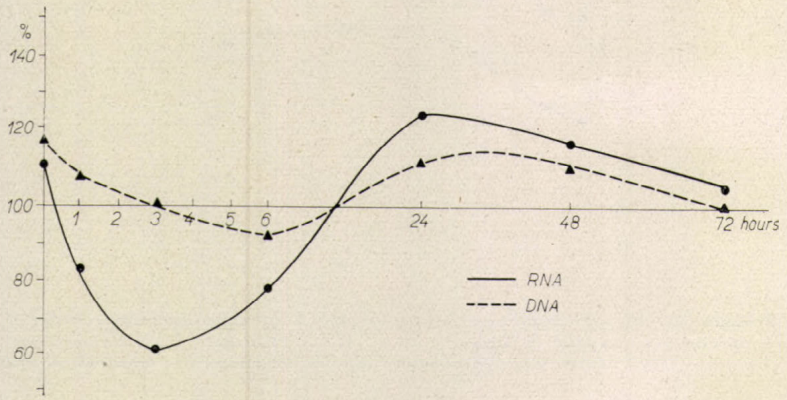

Fig. 4. Changes in the nucleic acid content of the skin (mice) after mechanical injury (15 kg/cm²). Abscissae — hours; ordinates — relative changes

ribonucleoprotein complexes so that phosphate groups of nucleic acids are set free and the binding of basic dyes is strongly increased (first or basophilic phase of injury).

These changes in the state of cell nucleoproteins are especially striking in severe irreversible injuries and can be demonstrated using appropriate methods. We have found that the difference between normal and mechanically injured cells is not influenced by some fixatives, the best being acetic alcohol. After such a fixation and staining the tissue slices in Unna's methyl green-

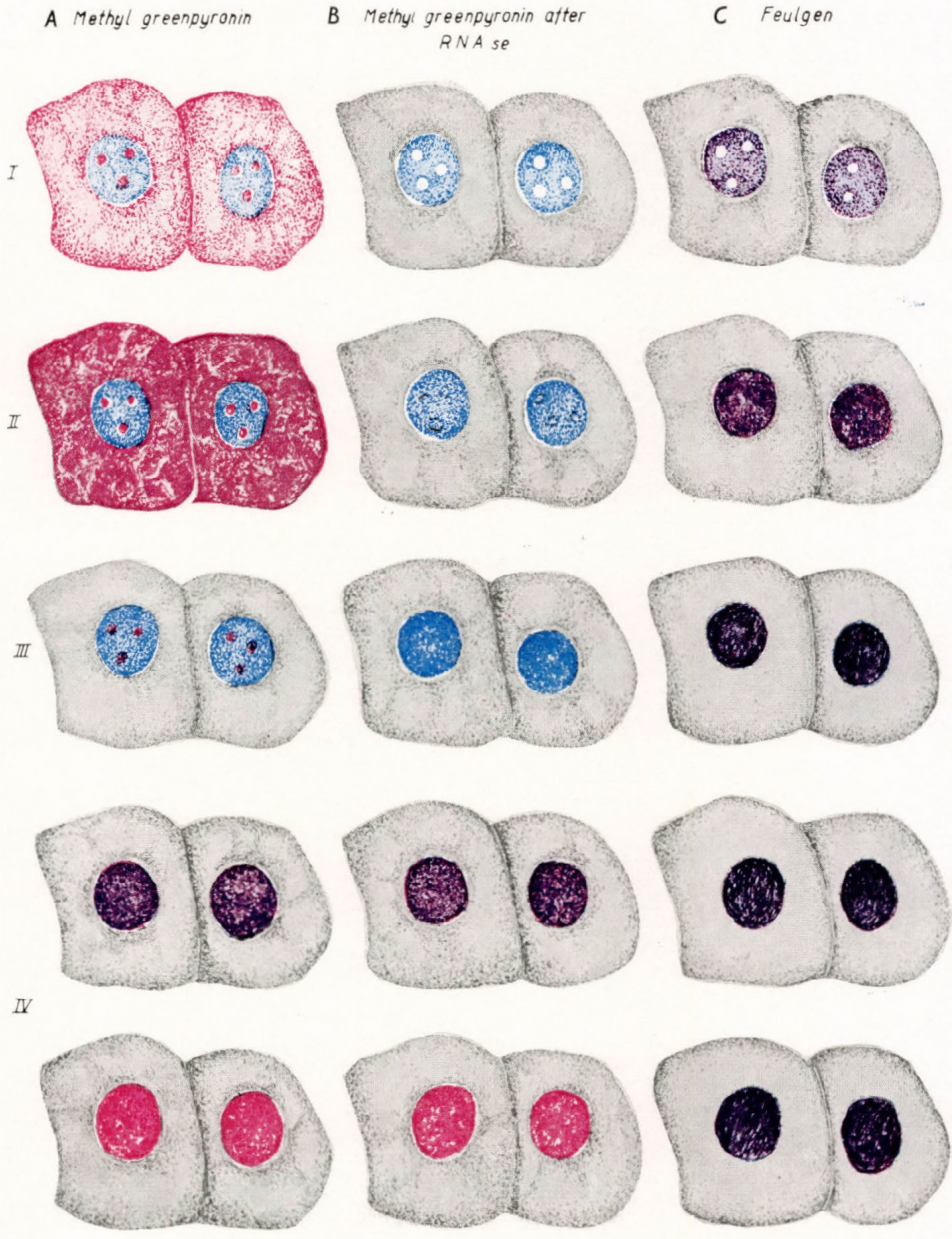

Fig. 5. Cytochemical changes in nucleic acids after irreversible mechanical injury. *A* — Methyl green-pyronin; *B* — Methyl green-pyronin after ribonuclease treatment; *C* — Feulgen. *I* — Normal cells; *II*, *III* and *IV* — First, second and third phases of injury

pyronin (Grübler), the pyronin is loosely bound to RNA in such a way that after alcohol treatment the cell protoplasm remains completely colourless. When the same procedure is applied to tissues which were injured by a pressure of 15—20 kg/cm² the pyronin is strongly bound to RNA and cannot be extracted with alcohol. This method makes the difference between affected and normal cells quite evident.

It should be stressed that irreversibly injured cells exhibit a similar increase in DNA basophilia. Since in this case there are also morphological changes in the nucleus, it is difficult to ascertain whether the increased dye adsorption is due to changes in the nucleoprotein structure or it is an effect due to a decrease of nuclear volume and the condensation of the solid phase of the nucleus.

The question arises here what after-effects result from these alterations in the nucleoprotein structure. Cytochemical studies on irreversibly injured cells after wounding have shown that the first basophilic phase of injury is soon followed by a second phase characterized by a complete lack of basic-dye adsorption in the injured protoplasm [53] (Fig. 12), which is marked about 3 hours after injuring the cells.

This important observation indicates that irreversibly injured cells lose their RNA after the latter has been set free from the ribonucleoprotein complex. Since only polymerized RNA exhibits affinity to basic dyes, the lack of basophilia suggests that a degradation of RNA takes place.

It is of interest to examine whether some degradation of RNA occurs also after a reversible injury. Brachet pointed out that increased basophilia after injury is followed by a decrease in the adsorption of basic dyes [7, 8]. In order to establish the quantitative changes in nucleic acid content after reversible injury, we have made estimations of RNA and DNA content in mouse skin which has been subjected in vivo to a mechanical pressure of 10 to 15 kg/cm². These experiments [49] proved a decrease of the RNA content as early as the first hour whereas no significant changes in DNA could be found (Figs 2 and 4). The maximum RNA degradation was observed between the 3rd and the 4th hour after injury which is in good agreement with cytochemical observations.

When cells are killed by irreversible injury, the nuclear DNA undergoes some changes too and several hours after the complete degradation of RNA, exhibits some signs of depolymerization [42]. DNA adsorbs intensively pyronin instead of methyl green giving a marked Feulgen reaction. This phenomenon according to Kurnick [30] is characteristic of depolymerized DNA.

The following scheme (Fig. 5) shows in general the cytochemical changes both of RNA and DNA in irreversibly injured cells.

Nucleic acids and cell recovery after reversible injury

In order to elucidate the growth-promoting mechanism in wound healing we followed up the reaction of tissues to slight injuries which do not kill the cells. Such injuries may be obtained by applying a properly determined mechanical pressure on a skin flap of the animals by a special device [60]. In these experiments we have established [59, 60] that the epidermic cells of mice are reversibly injured by pressures between 5 and 15 kg/cm². Pressure higher than 15 kg/cm² produces cell-death, by pressure of 20 kg/cm² most of the cells are

killed. Lower pressure not being harmful to the life of epidermic cells proved to be an intense activator of cell proliferation. The stimulation of mitotic activity is limited within the skin area to which the pressure was applied (Fig. 6).

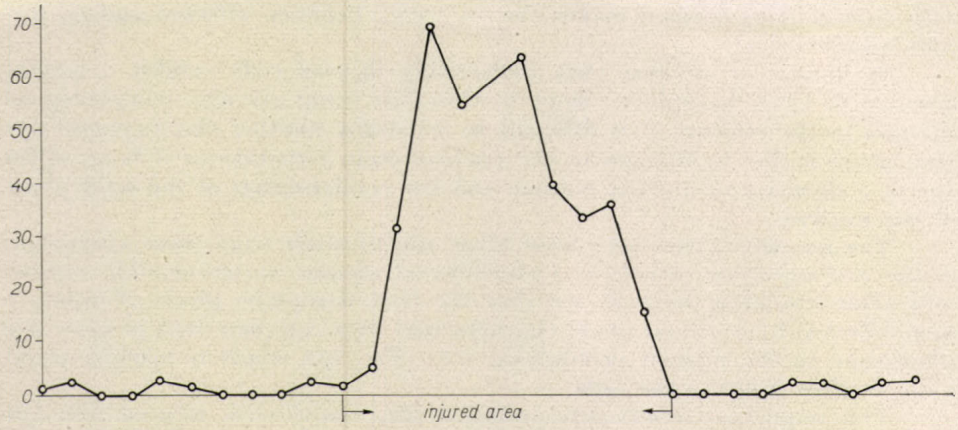

Fig. 6. Mitotic activity in epidermis (mice) 48 hours after mechanical injury (15 kg/cm²) [59]

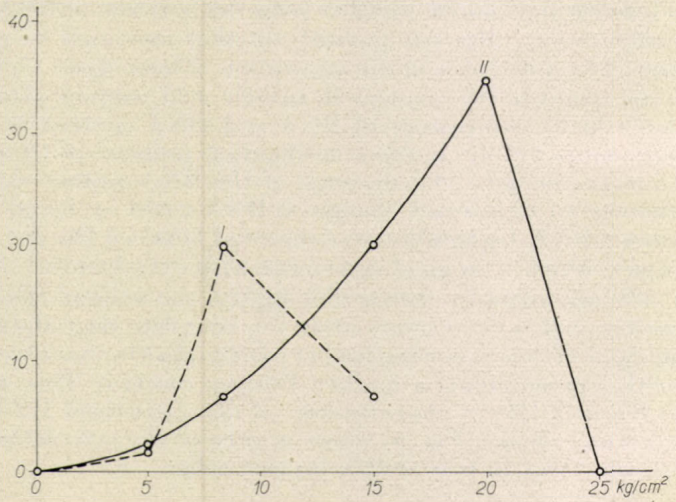

Fig. 7. Dependence of the activation of mitotic activity in epidermis (mice) on the mechanical pressure. *I* — After 24 hours; *II* — After 48 hours [59]

The degree of stimulation appears to be in direct proportion with the pressure applied (Fig. 7).

Biochemical estimations of nucleic acids in those cases when cell proliferation is activated by pressure ranging from 10 to 15 kg/cm² have shown that a new synthesis of RNA follows the period of RNA degradation. During the first 24 hours the initial amount of RNA is not only restored but also

increased by 20—30 per cent (Figs 2 and 4). It should be noted that the stimulation of mitotic activity begins during the period of intense RNA resynthesis (Fig. 8).

It is generally accepted that high RNA content is a necessary condition for cellular growth. The onset of cellular proliferation after a period of degradation and subsequent resynthesis of RNA makes it reasonable to assume that it is not the absolute quantity of RNA, but the synthesis of new RNA which is of importance for cellular growth. Such an idea was suggested in connection with experiments on bacteria, showing that the biosynthesis of proteins in bacterial cells is dependent on the synthesis of new amounts of RNA [27, 36, 37, 44, 45].

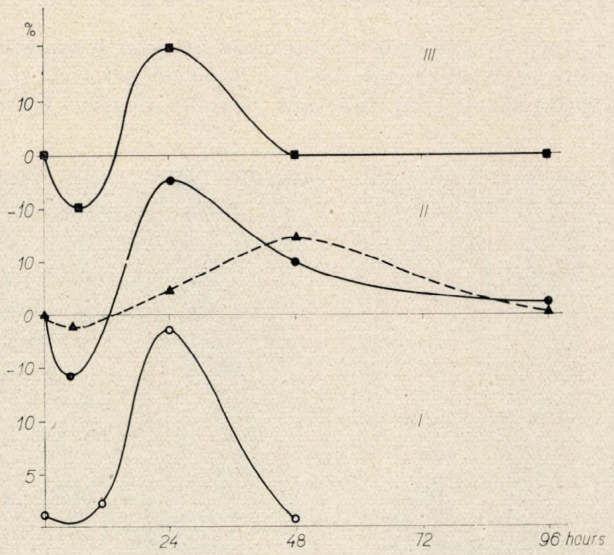

Fig. 8. Mitotic activity in epidermis and nucleic acid content of the skin (mice) after mechanical injury (10 kg/cm²). *I* — Mitotic activity; *II* — RNA (full line) and DNA (dotted line); *III* — RNA/DNA [59]

Our data render it very probable that partial degradation of RNA is an indispensable moment for the synthesis of new RNA. In such a way RNA degradation appears to be the stimulus for a regenerative process. This idea is supported by some data in the literature that a preliminary partial degradation of RNA with ribonuclease increase the incorporation of labelled amino-acids into proteins [25].

It was also found by Kramer and Straub [29] that the induced enzyme synthesis is stimulated by a preliminary treatment of the cells with ribonuclease. These authors assume that the degradation of RNA is a favourable process supplying nucleic acid precursors for the formation of a specific RNA involved in protein synthesis. Our observation that injury-induced cellular growth is also preceded by degradation and resynthesis of RNA suggests that this may be a general way of stimulating protein synthesis. We cannot give any details concerning the mechanism of this stimulation. Nevertheless, it is

evident that in this respect the greatest part of the intracellular RNA is metabolically inert. Our experiments with dye adsorption seem to show that this inactivity is connected with a strong interaction between the phosphate groups of RNA and some active groups of proteins. The degradation of this inactive RNA in cases of cell injury makes possible the synthesis of an active one with free phosphate groups.

It appears, therefore, that ribonucleoproteins are the injury sensitive unit of the protoplasm: their reaction to injuring agents is the starting point of regenerative processes.

Rôle of break-down products of RNA by irreversible injury

Irreversible injury affecting the nucleus leads to a complete degradation of RNA. It seems that in the absence of nuclear activity the formation of new RNA is inhibited, so that the cells cannot survive. During the wound

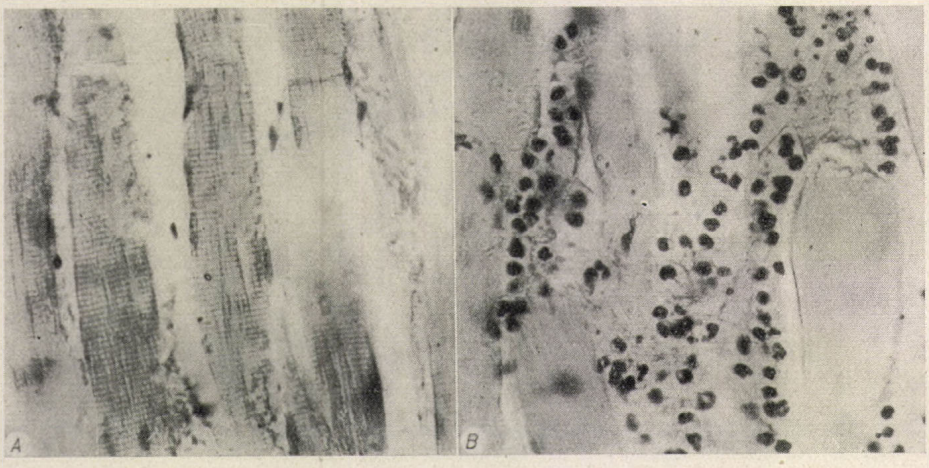

Fig. 9. Tourniquet injury of muscle tissue (mice). *A* — First phase; *B* — Second phase (RNA degradation). Methyl green-pyronin [56]

process these cells are demarcated and eliminated. Our investigations show that RNA degradation in injured cells plays an important rôle in these processes, too.

As is well known the demarcation of irreversibly injured cells is realized by means of a leucocyte infiltration forming a barrier between intact and dead tissues, the emigration of leucocytes in this case not being connected with microbial infection, but with irreversible injury of cells. It has been suggested that some leucotactic substances are liberated during cell injury (for review see [26]). Most of the authors accepting the theory of the injury-induced liberation of these substances, differ in opinion as to their chemical nature. Menkin has supposed that nucleic acid derivatives are set free during cell injury. His experiments, however, have given negative

results and this led him to accept the existence of a special substance, the so-called leucotaxine, which was assumed to cause emigration of leucocytes after tissue injury [34]. The natural occurrence of leucotaxine is very doubtful [38]. Other investigators have also assumed the liberation of nucleic acid derivatives during tissue injury, but so far have not succeeded in proving this [7]. More definite results were obtained by Alpern and Lipshitz [2] who

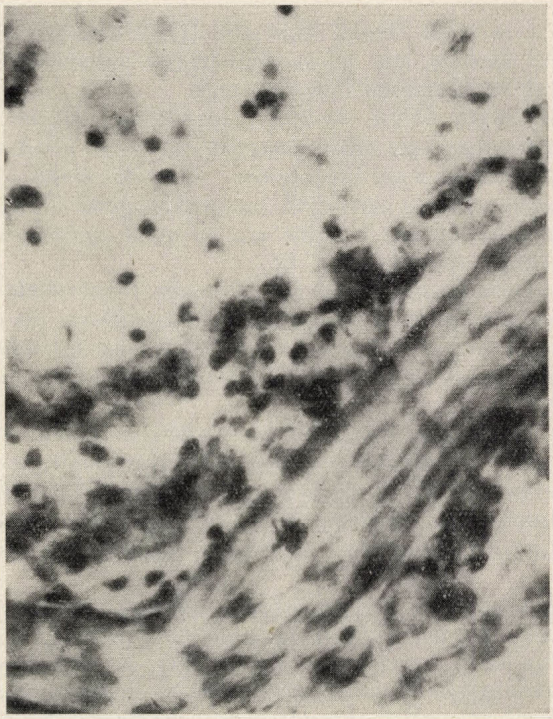

Fig. 10. Leucocyte emigration under the influence of RNA-hydrolysate [56]

presented data showing adenylic acid to be strongly leucotactic and accepted this substance as being the inflammatory factor set free by tissue injury. Paskhina [39], who failed to establish the presence of adenylic acid in inflammatory exudates disagrees with this statement. Contrary to the findings of Alpern, Japanese authors have reported adenylic acid as having a negative leucotactic effect [22].

Considering these contradictory findings, it is not possible to draw a final conclusion. As shown above, our data give strong evidence that in injured cells RNA is degraded during the first three hours. There is no doubt, therefore, that RNA break-down products are released by irreversibly injured cells. Through cytochemical observations we have established a very close relationship between RNA degradation in injured cells and leucocyte emigration. It was found that the degradation of RNA shortly precedes the emigration of leucocytes. The formation of a leucocytic barrier begins about three hours

after injury when a complete degradation of RNA in the injured cells has already taken place. The examination of the microscopic slices has shown that leucocytes accumulate around injured cells only after the latter have lost their RNA. An irreversibly injured tissue which has not yet undergone the phase of RNA degradation, is not leucotactic (Fig. 9). This is the reason why the formation of leucocytic barrier is limited by the area of RNA degradation, and in this way the irreversibly injured cells are demarcated from the rest.

Therefore, it is most likely, that the RNA break-down products released by injured tissues are those leucotactic substances whose existence had been assumed by previous investigators.

Experiments with RNA hydrolysates bring strong support to this statement. The subcutaneous administration of such RNA derivatives leads to an intense infiltration with leucocytes. As early as 30—40 minutes after introducing the nucleotides, a leucocytic emigration into the tissues is observed (Fig. 10). In this respect our results are in good agreement with the data of Alpern concerning adenylic acid [2].

The break-down products of RNA apart from their well established vasodilative effect also proved to increase the capillary permeability [2, 56] Intravenous introduction of trypan blue following an intradermic application of RNA hydrolysate leads to a deposition of the dye in the areas of hydrolysate injection.

Bearing in mind that the defensive function of leucocytes depends on their phagocytic activity, we have carried out experiments to clear up whether the leucotactic effect of RNA derivatives is linked with their influence on phagocytosis. Alpern and Lipshitz [2] have shown that adenylic acid apart from its leucotactic effect has a strong stimulating action on phagocytosis.

We have examined [62, 63] the influence of RNA hydrolysate on leucocyte phagocytosis *in vitro* and *in vivo*. Within some concentration limits a stimulation of phagocytosis in vitro was observed (Fig. 11). As is evident, concentrated solutions up to 10 mg per cent strongly inhibit phagocytosis. This inhibition decreases by diluting the solution, and lower concentrations ranging from 1 mg to 0.01μg per cent have a stimulating effect. The maximum increase of phagocytic activity is about 60 per cent at a concentration of 10μg per cent. Similar results were obtained by Ludány and Vajda with nucleic acids [32, 33]. The influence of RNA nucleotides on phagocytosis proved to be much stronger in vivo. Introducing these substances intraperitoneally, we have succeeded in increasing by 300 per cent the phagocytosis of blood leucocytes Compared with other substances activating phagocytosis nucleotides appear to be the most powerful stimulants of this process.

All these data support the concept that the inflammation process induced by injury (leucocyte emigration and phagocytosis) is set on by RNA break-down products released from the injured cells.

It should be noted here that all data mentioned concern only mammals. Some preliminary experiments in our laboratory show that the same holds true for birds, whereas in lower vertebrates the injury-inflammation cannot be related to RNA degradation [56]. This proves, therefore, that the influence of RNA break-down products on capillaries and leucocytes appeared through evolution in connection with the increasing reactivity of connective tissue.

As for this reactivity, the state of the nervous system plays an important rôle. Our experiments with narcotized animals, or animals having brain injury show [54, 55] that subcortical inhibition completely stops the emigration of leucocytes, although the body temperature was artificially maintained at normal level. In this case cytochemical studies have established that a degradation of RNA takes place normally, independent of the state of the nervous system (Fig. 12). It is not, therefore, to the lack of leucotactic substances, but

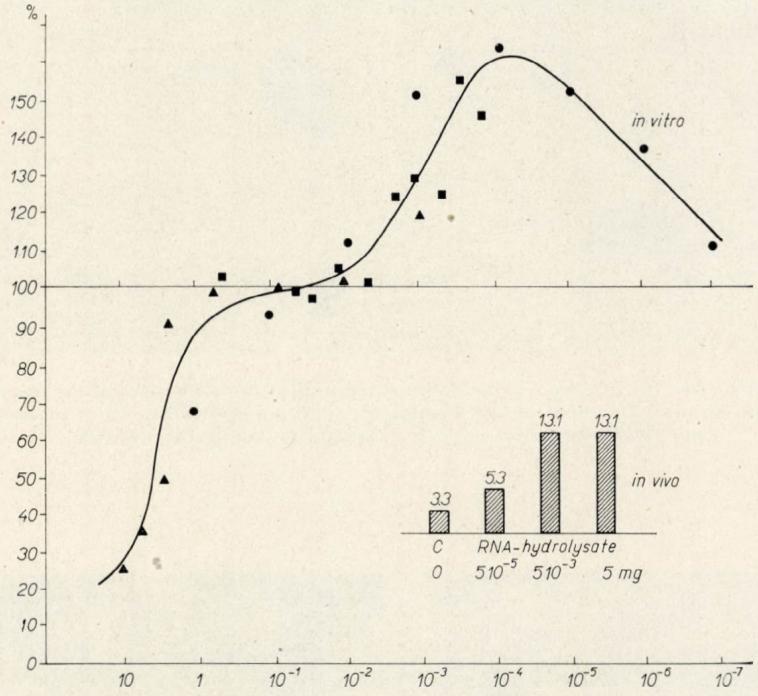

Fig. 11. Changes in phagocytic activity of leucocytes under the influence of RNA-hydrolysate in vitro (abscissae — relative changes, ordinates — RNA concentration) and in vivo (phagocytic numbers) [62, 63]

to the loss of tissue reactivity, that the inhibition of leucocyte emigration is to be attributed. This is in agreement with Alpern's experiments [3], that no influence of adenylic acid on capillary permeability and leucocyte emigration is observed, after local novocain anesthesia.

The importance of RNA break-down products for the wound process is also supported by the results from clinical observations on slowly healing wounds and ulcers treated with hydrolysate of RNA [61]. In 70 per cent of the cases a very good therapeutic effect was obtained. The favourable influence of nucleotides on vasodilatation, leucocyte emigration and phagocytosis resulted in increasing the biological cleaning rate of the wound and accelerating the formation of granulation tissue. Cytological studies of wound surface using the method of Makarov and Pokrovskaja confirmed the leuco-

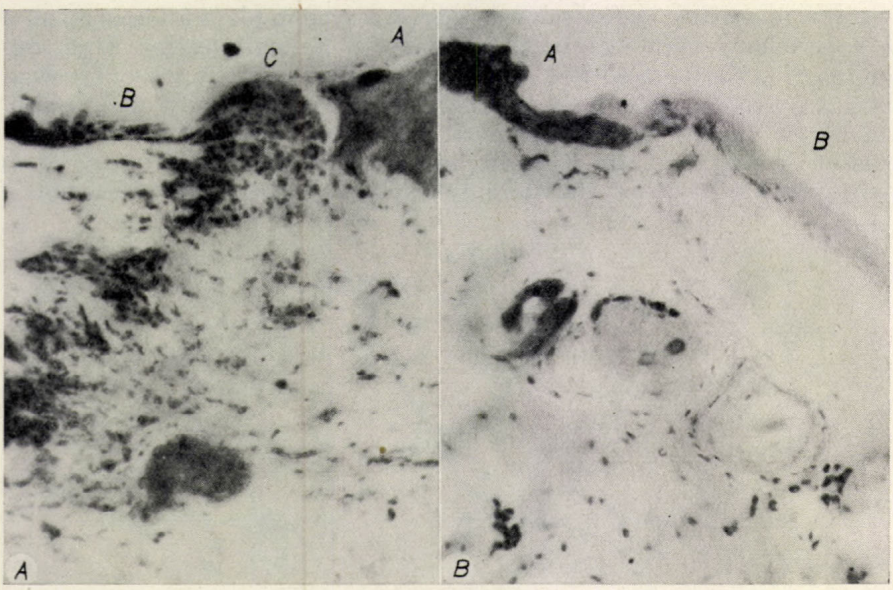

Fig. 12. Influence of narcosis on leucocyte accumulation after wounding (mouse skin). *A* — normal animal; *B* — narcotized animal; *A* — intact epidermis; *B* — injured epidermis (RNA-degradation); *C* — leucocyte accumulation [55]

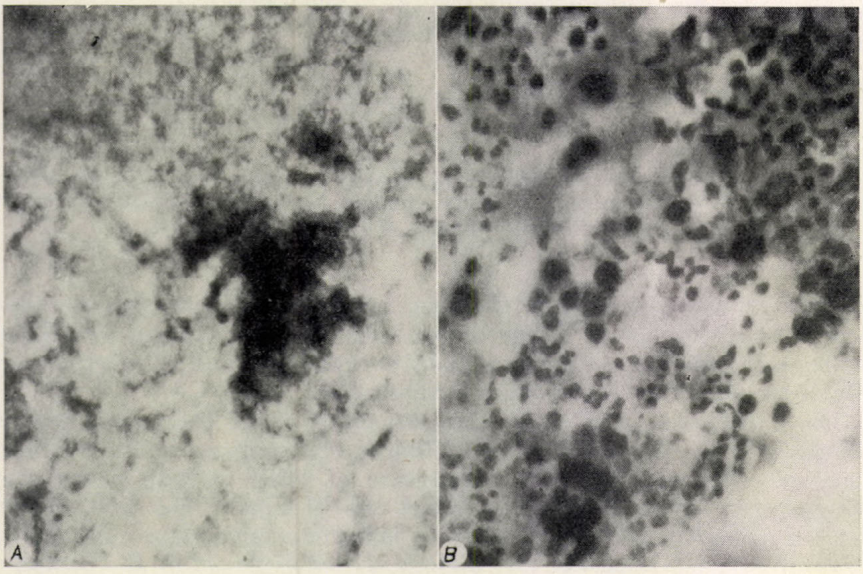

Fig. 13. Cytological picture of a varicose ulcer before (A) and 24 hours after (B) treatment with RNA-hydrolysate [61]

tactic effect of the ribonucleotides. In ulcers with absolute lack of leucocytes before treatment, a normal leucocyte infiltration was established as early as 24 hours after treatment with RNA hydrolysate (Fig. 13). Our findings are in agreement with some clinical observations concerning the effect of adenylic acid on ulcers and inflammatory processes [1, 8, 17, 47].

Possible mechanism of injury-induced RNA degradation

All data indicate that the protoplasm ribonucleoproteins have a central position in the cell response to injuring agents. Therefore, it would be of importance to elucidate the mechanisms involved in the RNA degradation. There is no doubt that the mechanical pressure itself cannot degrade RNA. During in vitro experiments pressure as high as 1000 kg/cm^2 did not result in degradation of RNA [64], whereas in vivo 15 kg/cm^2 was quite effective.

Two main speculations may be discussed: either the injury inhibits synthetic processes, thus affecting the balance between normally occurring degradation and synthesis of RNA, or the injury in some way stimulates the RNA degradation.

The former hypothesis is rendered less likely by the fact that a complete degradation of RNA in injured cells can be observed in a very short period of about 3 hours. Such a high rate of RNA turnover is not probable, bearing in mind that an approximate "half-life" of about 8 days was calculated for adult liver RNA [6].

The second hypothesis that RNA degradation is stimulated is more probable, and can be related to recent findings, that microsomal ribonucleoprotein particles have considerable amounts of ribonuclease activity [18, 20, 42, 43, 46, 49, 51]. In *E. coli* the ribonuclease was found to be in a latent state, the separation of RNA from the protein moiety initiating the enzymic activity of the latter [20]. Experiments with ribonucleoprotein particles isolated from animal tissues suggest that they probably contain all the ribonuclease in an active state [43, 49, 51]. Nevertheless, this activity was to some extent activated by urea solutions releasing RNA from the ribonucleoprotein complex [51]. On the other hand, tissue injury may also result in damaging the lysosomes which contain an acid ribonuclease liberated under the influence of many physical and chemical factors [5]. In intact cells the stability of RNA in the presence of intracellular ribonucleases was suggested to be due to the protective effect of the protein moiety of ribonucleoproteins. It is very probable, therefore, that the separation of RNA from this complex might result in a degradation of the RNA, due, either to the activation of a ribonuclease activity, or to the removal of the protective influence of the proteins, the two possibilities not being alternative. In this way in case of tissue injury the separation of RNA from the proteins and the activation of intracellular ribonucleases would explain the resulting degradation of RNA.

Some data indicate that mechanical injury results in changes of the intracellular liquid phase. Determinations of the dry residue and nucleic acid contents of the skin, immediately after mechanical pressure of 5 to 15 kg/cm^2, showed an increase in dry residue of about 15 per cent, and an increase

in both RNA and DNA contents of about 10—15 per cent. These data prove quite clearly that at the moment of injury the affected tissue loses water, as well as water-soluble substances. In connection with this observation the extreme lability of nucleoproteins, as well as the rôle of the ionic environment for maintaining the structure of ribonucleoprotein particles, should be mentioned [24, 40, 50].

It is very likely, therefore, that mechanical injury affects the nucleoproteins by producing changes in the distribution of soluble substances which is important regarding the structure of ribonucleoproteins. The biological significance of the latent ribonuclease activity, considered in the light of our results, becomes quite clear. In irreversible injury the ribonuclease activation realizes the supply of leucotactic substances for demarcation of necrotic tissues. In reversible injury it leads to a partial degradation of RNA, in this way activating the cellular growth. Such a rôle of the ribonuclease activation in tissue injury would be in agreement with some data indicating a close relationship between ribonuclease activity and cellular growth [13, 14]. This makes very probable the suggestion of Elson [20] that perhaps this enzyme plays a special rôle in protein synthesis.

On the basis of these data we shall attempt to explain the starting mechanism of wound healing. Mechanical injury, which is the most usual cause of wounding, is the result of a high mechanical pressure killing the cells. As was established for mice epidermic cells this pressure should be higher than 15 kg/cm^2. Having in view the physical properties of tissues we must pay attention to the fact that an injuring force acting as a perpendicular pressure on the tissue at the same time produces a lateral gradient of mechanical pressure rapidly decreasing from the focus of the injury. As a result three different concentric areas are formed: 1. Necrotic area; 2. Area of reversible injury; 3. Adjacent intact area.

In the first area the cells are killed and complete RNA degradation takes place within a few hours, the DNA being affected, too. This area, therefore, is the main source of the leucotactic RNA break-down products.

The second area of reversible injury caused by the laterally propagated pressure wave is spread out within the limits of pressures killing the cells and those which are too low to cause degradation of RNA, *e.g.* in the case of mouse epidermis between 15 and 5 kg/cm^2.

In this area the cells survive and undergo the so-called dedifferentiation which is closely connected with cell proliferation. The calculation of mitotic rate in this area of the epidermis shows that a maximum of mitotic activity is established at a certain distance from the necrotic area (Fig. 14) [53]. Such a mitotic gradient around the wound was also established in lower vertebrates [19] as well as in cultured in vitro mouse ear [23]. When this distribution of mitosis around the wound edge is compared with the dependence of mitotic stimulation on mechanical pressure (Fig. 7), a good parallelism is observed. It is most likely, therefore, that the particular distribution of mitosis after wounding merely reflects the lateral pressure gradient of the injuring force which leads to a reversible RNA degradation in the affected cells. All this can be illustrated by the following scheme (Fig. 15).

It should be noted that other factors also contribute to these reversible changes in the second area; one of these is the disturbed blood circulation. Experiments with periodical stoppage of blood circulation [58] reveal similar

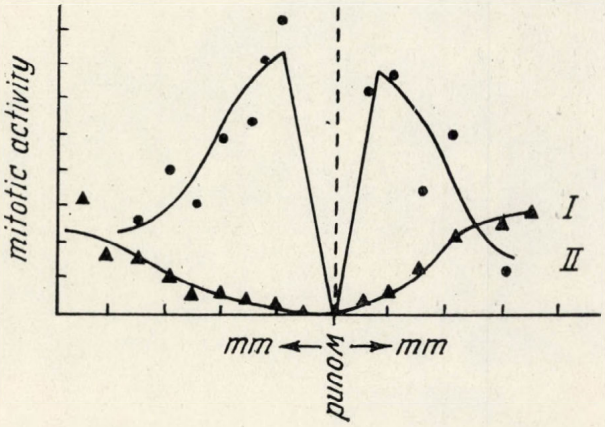

Fig. 14. Distribution of mitotic cells around the wound (mouse skin). *I* — 6 hours, *II* — 24 hours after wounding [53]

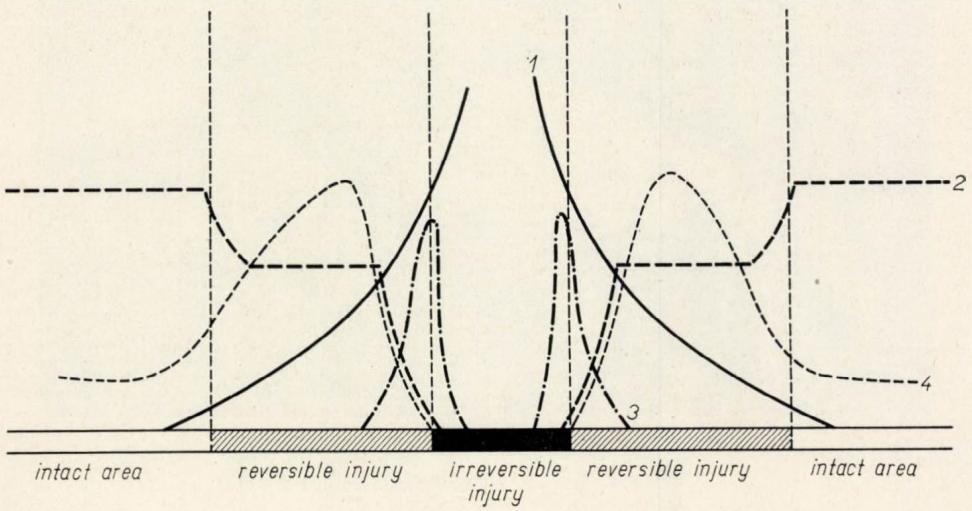

Fig. 15. Scheme of the injury-induced changes in tissues. *1.* Mechanical pressure at the moment of injury; *2.* RNA content after 3 hours; *3* — accumulation of leucocytes after 6 hours; *4* — mitotic activity after 24 hours

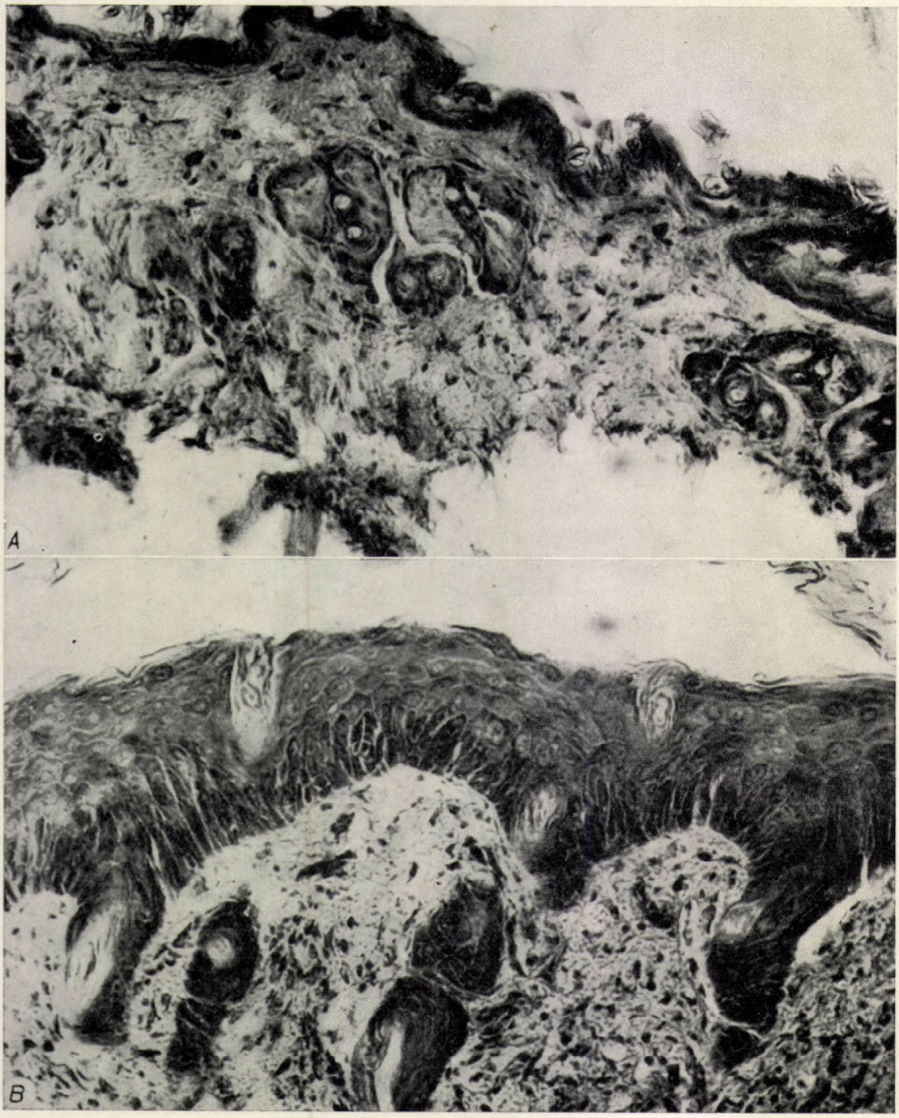

Fig. 16. Epidermis hyperplasia after periodical stoppage of blood circulation (mice). *A* — Normal, *B* — Hyperplastic epidermis [58]

changes in cell ribonucleoproteins, as after mechanical pressure. At the same time a strong stimulation of cellular dedifferentiation and proliferation is observed (Fig. 16) [58].

In the light of these results we assume that the regenerative processes in the area of reversible injury are promoted by RNA degradation, induced by mechanical pressure and by slight temporary disturbances in the blood supply.

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Contributory Discussion

Prof. Krompecher: You mentioned that the blood supply was periodically interrupted in the last experiment.

How did you perform this?

Dr. Tsanev: The leg was ligated with a rubber ring for three hours every day. After three or four days the proliferation can be observed preceded by a degradation of RNA.

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COMPARATIVE STUDIES ON THE DYNAMICS OF REGENERATION IN THE COURSE OF WOUND HEALING AND GROWTH OF SKIN CANCER

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Synopsis

During the healing of a circular wound, 1 square cm in size, made in the skin of a rat, the crust covering the surface is detached and is again formed three or four times in succession. Microplanimetric measurements have shown that the size of the necrotic tissue is several times greater than that of the proliferating epithelium. When the crust is exchanged, the newly formed epithelium becomes detached, then grows again from the wound margins. At certain times, but especially in the course of impaired repair, the epithelium may grow deep broom-like, into the underlying tissue, in a way which resembles infiltrative growth. In the course of pathological wound repair the growth of the epithelium into deeper layers is particularly significant, but on the papillae thus growing the cells show phenomena indicative of differentiation. In the early phase of their effect, carcinogenic substances give rise to a hypertrophy of the epithelium. The surface becomes keratinized and is desquamated over and over again. At the onset of papillomatous and carcinomatous growth, masses of keratinized and necrotic tissues are shed off. In the centre of the deep growing epithelial papillae the epithelial cells become cornified, most of them perish; in these areas and in their environment the contact between the epithelial cells loosens. The whole mass of epithelial cells breaks through into the connective tissue and spreads all over the organism. In the course of carcinogenesis epithelial defects can be found, which swiftly repair. According to the experiments of Deelman this reparative process may have a cocarcinogenic effect, *i.e.* it may promote the development of cancer. The keratinized and necrotic centres of the deep-growing epithelial papillae may create epithelial defects as they are growing into the connective tissue which may have significance in the initiation and continuance of invasive growth. Later, the deep-growing papillae of cancer become anaplastic and usually show no differentiation. It is probable that from the perishing tissue substances are released that may have a significance on the permanent proliferation of the epithelium; however, reliable evidence as to this point is not available yet.

Introduction

According to many authors, various kinds of similar features and correlations exist between regenerative and tumorous growth.

Fischer-Wasels [4] claims that every malignant tumour develops on the basis of some regenerative process. Deelman [1] made a wound in the skin area previously painted with tar or with carcinogenic substance. In those areas carcinomatous growth begun more often and much earlier, which is usually explained by assuming that regeneration has a cocarcinogenic effect. The biochemical changes taking place in the embryonic regenerative growth and in tumorous growths resemble each other in many respects [7]. In view of these data it is understandable why many authors try to measure the therapeu-

tic effect of compounds on neoplastic growth by studying their inhibitory action on regenerative growth [6, 7, 8, 9].

In the present investigations we have compared the morphological changes taking place in the course of wound healing with the morphogenesis of cancer of the skin induced by treatment with methylcholanthrene.

Materials and methods

Using a trephine, two epithelial defects of equal size (1 square cm each) were made in the centre of each rat's back. The repair of these wounds was followed up daily by taking photographs and by studying histological preparations.

The dynamics of carcinogenesis was studied on mice. The interscapular area was painted twice a week with 20-methylcholanthrene (0.5% in acetone). Histological sections of the whole area of the painted skin were made weekly to observe the dynamics of the morphological changes.

The animals were examined twice a week, and changes were recorded and photographed at very short intervals. In general, a diffuse hyperplasia was found on the 10th to 20th day, and papilloma on the 30th to 40th day. In some animals neoplastic growth began as early as the 90th day, whereas in others it began after 180 to 200 days. From the histological preparation, as a whole, we took an overall picture with a magnification of 4 to 6 times, of which we made copies 20 to 30×40 to 50 cm in size, with a magnification of 5 to 6 times. In these we delineated the areas to be scrutinized (crust, epithelium, necrotic parts, areas showing various types of carcinomatous growth, etc.) with a microscopic control of the original preparation, then the areas thus delineated were measured by planimetry.

Results and discussion

It is well known that cytostatics retard the wound healing process. Qualitative and quantitative evaluation of this process has been made to apply the method for assessing the effectiveness of new compounds. According to histological studies these agents invariably produce morphological changes. However, the planimetric values for wound sizes varied, over a very wide range, when the measurements were made at a certain time (for example on the 10th day), but great variations occurred also during the time of repair. For these reasons, evaluation was more difficult then when studying tumours [2, 3].

Nevertheless, these follow-up and comparative studies yielded some remarkable evidences.

The surface of the wound inflicted, as specified above, is covered by an enormous crust which is cast off and formed again three or four times in the course of wound healing. The offcast crust is mainly composed of connective or granulation tissue, blood, dried serum and clot, and of round cells. However, the cast off crust also contains most of the newly formed epithelium. Thus, the regenerating epithelium perishes over and over again, then, after a short resting period, it creeps forward again from the wound margin in the form of a narrow line of cells.

The morphogenesis of skin cancer induced by treatment with carcinogenic hydrocarbons begins with an initial epithelial hyperplasia, which leads to papilla formation. The onset of carcinomatous growth is at first only marked by a polymorphism of the cells and by the beginning of invasive growth; the epithelium is similar in structure to the basic tissue, skin, in this case, and

shows organotypic growth. In the course of infiltrative growth bundles of woven, reticular structure penetrate into the underlying tissue which are composed of undifferentiated cells. Finally, the formation of pericarcinomatous metastases begins [5].

Already on the onset of proliferation, at the beginning of the hypertrophic phase, the surface is covered by a cornified, necrosed, crust-like mass. Throughout the growth of papilloma or epithelioma we find large masses of

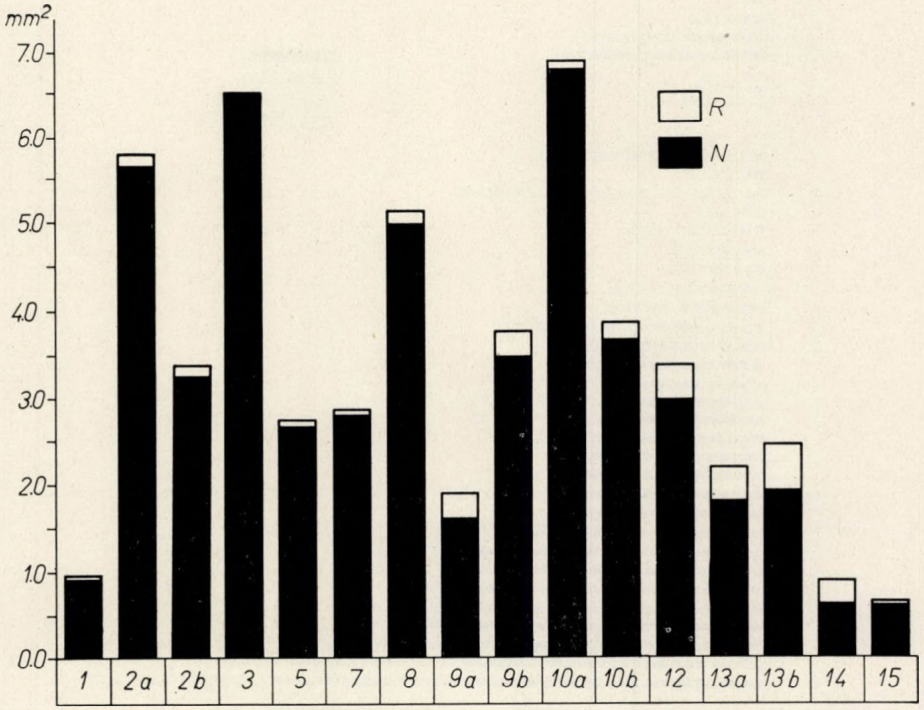

Fig. 1. Healing of the wound (1 cm²) made with trephine in the back skin of a rat. *N* — necrotic, crusty areas. *R* — epidermis formed in the course of wound healing, in square mm. Below: time in days

necrotic epithelium and connective tissue, resembling the crust not only in structure but also in that they are cast off and reproduced again and again. The centre of the tumorous papillae penetrating into the underlying tissue becomes, cornified, the contact between the perishing cells is loosened, and in this way pseudolumina are formed. At some points disjoined epithelial cells break through toward the connective tissue (Fig. 3 *C*), invading the tissue interspaces, lymphatics, capillaries. Thus, the disintegrating parts of the ingrowing papillae spread throughout the organism, but not outwardly. It is probable that from the crust, and also from the cancerous tissue, degradation products are constantly released in dissolved state.

The results of the microplanimetric measurements illustrate even more clearly (Figs 1 and 2) that in the course of wound healing the amount of epithelium is negligibly small as compared with that of the tissue showing crusty

necrosis. Likewise, in the course of tumorous growth the amount of keratinized and necrotic mass exceeds by far that of the florid tumorous epithelium. We find small quantities of necrotic tissue only if for some reason the crust was cast off prior to histologic study. Thus, in the course of these two processes

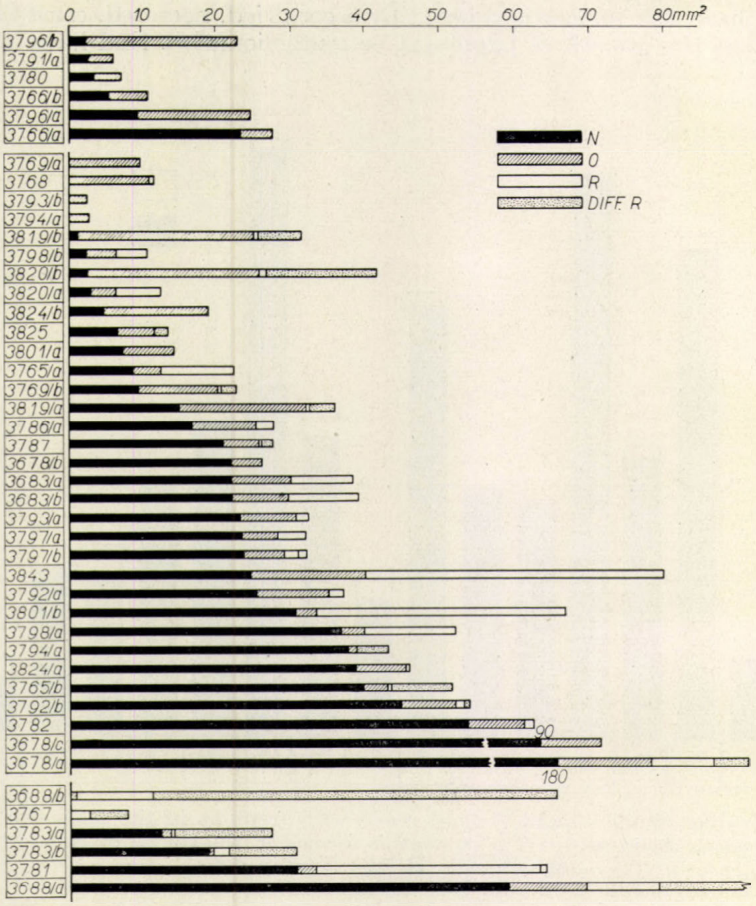

Fig. 2. Cancer of the skin, induced with methylcholanthrene in the back skin of a mouse. *N* — cornified, necrotic area. *O* — area showing differentiated, organotypic growth. *R* — area showing undifferentiated, reticular growth. *Diff. R* — area of reticular structure, showing secondary differentiation, in square mm

the epithelium perishes rapidly, *i.e.* the cells have a short life span and are swiftly exchanged.

In the following paper [8] we shall describe in more detail, that in the course of wound healing and in the initial phase of tumorous growth the newly formed epithelium is always differentiated. When cancer infiltrates underlying tissues by invasive growth, differentiation does not take place and the epithelium becomes anaplastic. This undifferentiated epithelium invades the connective tissue in the form of thinner or thicker bundles. During regener-

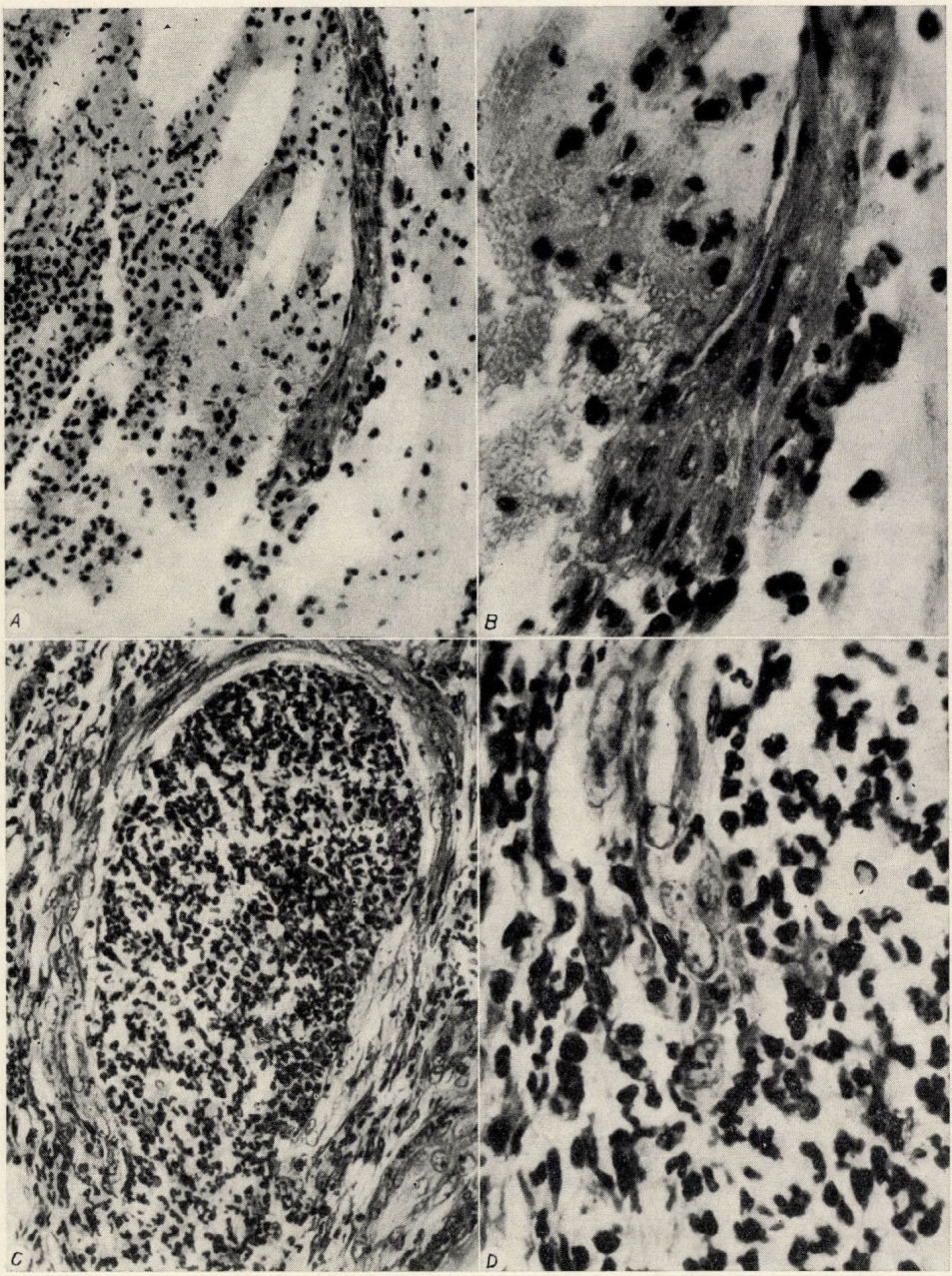

Fig. 3. *A* — Broom-like ramification of epithelium creeping forward under the crust. On the left: crust. On the right: connective tissue, on which the epithelium creeps along. ×350. *B* — The cells are rather well differentiated, tonofibrillar structure is visible in the high-power view. × 900. *C* — Islet of carcinomatous squamous cell epithelium growing deep. In the centre there is a pseudolumen, containing cornified epithelial cells among round cells. In a wide area the disintegrating mass has broken through toward the connective tissue and the disjoined tumour cells pour freely into the stroma. ×350. *D* — High-power view of the same area. ×900. At the site of the break-through an anaplastic tumour cell line, reminiscent of wound lips, is proliferating toward the connective tissue

ation it could repeatedly be observed that the epithelium creeping forward begins to show a broom-like ramifying growth (Fig. 3 *A* and *B*). In pathological wound healing [*e.g.* in varicose (crural) ulcer or fistula] it often occurs that the epithelium penetrates underlying tissues in the form of irregular, not cornifying lines of cells or bundles. This regenerative epithelial proliferation apparently showing invasive growth closely resembles squamous cell cancer. A serious diagnostic problem is encountered in differentiating one from the other. However, the investigations indicate that in such cases the epithelial cells exhibit the clear-cut signs of differentiation, whereas the anaplastic tumour cells show no signs of differentiation [8].

When the skin is painted with a carcinogenic agent, the epithelium usually shows hypertrophy, though small ulcers may quite often be formed, too. These ulcers are soon repaired by the epithelial cells growing inward from the wound margins. We have already mentioned that according to the experiments of Deelman the regenerative epithelial proliferation by a co-carcinogenic action accelerates the development of cancer. The epithelial elements and the perishing cells in the centre of the tumorous papillae likewise create epithelial defects when they invade the connective tissue. In those areas the epithelium creeps into the adjacent connective tissue in the form of a thin line of cells (Fig. 3 *C* and *D*). The ulcers that arise during carcinogenesis may explain why cancer develops far more rapidly in one animal than in another. And the proliferation of the epithelium of the ingrowing pegs may play a significant role in the initiation of invasive growth and in its becoming permanent.

Already at the end of the last century it was assumed that substances initiating proliferation of adjacent cells might be released from the disintegrating cells. Carrel attributed this role to the polypeptides and called the substances trephons. English and Bonner suggested that a "traumatic acid" might be formed [7].

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Contributory Discussion

Dr. TSANEV: I may mention that in our laboratory we have carried out many experiments on wound healing in connection with normal and malignant growth. In two points we are not in agreement with the experiments of Professor Kellner. First of all the question of necrosis in regenerating epidermis. Under normal conditions of wound healing we never observed repeated necrosis of regenerating epidermis. Only under pathological conditions when chronic inflammation occurs is the necrosis of regenerating epidermis observed. Old authors observing a necrotic layer of epidermis covering the wound surface stated that it is the first phase of regeneration, then this epidermis degenerates and the second phase is the undergrowth of epidermis. Russian authors, however, have shown that only the injured one damaged during wounding degenerates and it remains one or two days covering wound surface. The regenerating epidermis grows then under the necrotic tissue. In histological preparations one can obtain the whole necrotic layer and below it the undergrowing epidermis. After the first days this necrotic epidermis degenerates. Degenerating epidermis can be observed later only in pathological cases. That is why I cannot see any analogy between cancer growth with necrosis, and a normal wound healing process. In some observations in whole body X-irradiated animals the formation of granulation tissue was completely inhibited. Nevertheless, the regeneration of epidermis takes place and this epidermis takes the eliminative function of leucocytes. Concernig tissue and cell dedifferentiation, we tried with repeated injuries and repeated local anoxaemia to produce cancer-like proliferation in the skin of mice.

Prof. KELLNER: What was your method?

Dr. TSANEV: It was periodical stoppage of the blood supply and injuries. We have obtained a strong tissue dedifferentiation and a strong cell proliferation, but we did not succeed in producing such cancer-like growth. There was a growth of the epidermis and of the tissues but there was no such growth which can be termed like cancer growth.

Dr. LEHOCZKY: What will happen if one is dealing with chronically detached instead of intact epithelium as initial material?

Prof. ROMHÁNYI: The question whether an infiltrative mechanism has the same essential features in both regenerative and tumorous processes is of fundamental importance. The mechanism is similar whether one is dealing with tumorous infiltration or, in cases of wound healing, with epithelial infiltration, and the fundamental problem is why it is arrested here and unrestrained there. What is it that releases fibroblastic proliferation and why does it stop in cases of wounds and why not in other pathologic processes? The electron-microscopic arrangement of squamous epithelium is noteworthy: the peculiar teeth of its indented wavy cell membranes fit into one another. Cellular contacts ought to be studied in more detail. There exists a theory in connection with tissue cultures according to which the contact between tumour cells is loose and that infiltration is due to this phenomenon. Cells are provided with constantly moving membranes, and this is why even normal cells show infiltration in both tissue cultures and granulation tissue. Cells are architecturally and histologically "at rest" under normal histological conditions, a situation which, for endogenous causes, is not present in tumour cells. Normal fibroblasts come to rest after some time, and this stops further infiltration, whereas the undulating movement of sarcoma cells continues even after they have come into contact. The question, therefore, is how the products originating from these necrotized spots are able to influence movement and cellular contact.

Dr. CSABA: Repeated detachment of the scab presents, according to Prof. Kellner a parallel to the horny structures obtained in the course of carcinogenic painting. Carcinogenic treatment gives rise to epithelial hyperfunction, and it is precisely owing to this hyperfunction that epithelium, in the form of a horny structure, accumulates and becomes detached. Scab, on the other hand, is no product resulting from hyperfunction and cannot be regarded as having been brought about by differentiation. The two must be different. Another problem is whether the deeply lying necrosis should invariably be regarded as keratoid matter or only in the initial phase of proliferation. Has the keratoid nature of the substance been proved chemically and histologically? The third problem is the analogy concerning the formation of downgrowing epithelial columns and the broomlike branching of the migrating epithelium in cases of wound healing. Although the morphological picture and the tonofibrillar structure seem to allow the establishment of a certain analogy, I am not sure that it is justified since epithelial columns grow downward and open in the deeply lying connective tissue, whereas epithelium spreads over the surface in cases of wound healing, and it is during this process that the above-mentioned broomlike structures develop. While epithelial growth has the function to cover the surface of the wound

when the latter is healing, the opposite occurs in carcinomatous processes where the epithelium grows downward and its function is not that of covering surfaces.

Prof. TOKIN: Although I am no oncologist, our experiments in connection with regeneration included also investigations concerning blastomatous growth. It is imperative that we should carefully study histogenetic processes occurring in the course of regeneration and tumorous growth. The observation, that injury combined with carcinogenic treatment is especially favourable for the development of cancer, is highly interesting, and I should be glad to know Prof. Kellner's views in this respect.

As regards my own work, I want to say the following. My theory anent regeneration compelled us to concern ourselves with tumours. Processes of regeneration and blastomatous growth should, I think, be regarded as mutually antagonistic. We provoked carcinoma and sarcoma in our experiments by means of diverse cancerogenic agents, and regeneration in the tumorous zone always proved to be subnormal. Skin in the vicinity of spontaneous mammary carcinoma in mice loses its regenerative power. Adequate doses of chemical or physical cancerogenic agents inhibit regeneration, although — if administered in small doses — they might temporarily produce the opposite effect. However, regeneration is inhibited if the dose becomes large enough to induce tumorous growth. What does this mean histologically and cytologically? While we are not in a position to define tumour cells cytologically, our notions regarding histological occurrences are somewhat clearer. Skin, for example, represents an intricate complex of histological correlations. Epithelium may offer the picture of blastomatous growth if, for example, the subjacent connective tissue disintegrates. I think, therefore, that tumorous processes do not occur unless the tissues are systematically disintegrated by some endogenous or exogenous agent, in other words, tumours arise when normal integration is disturbed and the laws governing normal histology are upset. The masses of newly produced cells come promptly under the correlative influence of the remaining organ part. Regenerative blastomas represent in this phase a system of already correlated cells, and their further development continues in transplants and explants. No system of integrated cells develops in cases of tumorous growth: cells are either destroyed or begin to grow blastomatously, anarchically. Tumours do not, therefore, possess normal histological correlations. Generally speaking, tumours are not likely to develop in organs and animals where regenerative power is satisfactory and vice versa. No experimental tumours can, for instance, be produced in the limbs of amphibians. It is impossible to obtain tumorous growth in quite a number of lower animals *(e.g.* in sponges) because disintegration induces somatic embryogenesis in them.

Prof. KELLNER: Dr. Tsanev is skeptical regarding the periodic epithelial growth as observed on our object. It is on the evidence of observations made in the course of four years that we affirm that epithelial growth is not uniformly continuous: epithelium begins to grow, invades the crust, sloughs off, and grows anew. Like in the first instance, a protuberance is formed at the site of the sprouting epithelium after the detachment of the crust; the epithelium spreads out once more from the direction of the protuberance, disappears once more in the new crust, and this process is repeated three or four times. The phenomenon is reproducible, and its existence cannot be doubted. It is as good as impossible to examine the matter in any other form. It is not possible to examine the healing of wounds on the skin of land animals in a moist milieu. We tried to study the matter on frogs which were in water: instead of drying up, a small area of the skin surface became necrotic, this area was cast off, and the process was repeated several times. Human epithelium invariably shows surface necrosis in connection with the healing of wounds. The newly produced epithelium is cast off when necrosis sets in, and growth begins again and again. All our attempts to study the healing of wounds, human or non-human, otherwise than subcrustally were in vain. This applies to ulcus cruris as well which we studied in different phases. I shall revert to this matter. Although the surface does not display a large mass of necrotized matter but only a comparatively small necrotic area, it is beneath this area that the epithelium spreads forward, and the covering layer is then cast off anew. I should like to know the object on which it would be possible to eliminate inflammation. I think this process is released precisely by the necrotic mass itself.

Dr. TSANEV: My opinion is that the normal process of wound healing is not discontinuous. Without necrosis the regeneration of epithelium goes on without interruption. We have carried out our experiments on mice, under a crust. There were the same conditions only you have worked on rats. On inflammation maybe there is a misunderstanding, what you call inflammation. The leucocyte infiltration which is always to be observed on inflammation is not a pathological process. We have followed the regen-

eration of epidermis every day every two or four hours and we could not observe such a necrosis of epidermis.

Prof. KELLNER: Dr. Tsanev ran an exceedingly intricate experiment in which the formation of connective tissue was inhibited by intensive irradiation. We duplicated his experiments on animals that were irradiated, treated with colchicine, podophyllin, etc. Such manipulations inhibited the growth of granulation tissue to a certain extent, but by no means as vigorously as it would have followed from Dr. Tsanev's remarks. Although we obtained a certain degree of inhibition, and the quality of the granulation tissue underwent a certain change, what I regard as essential — and this is meant as a reply to Dr. Csaba also — is that differentiation is, in my opinion, no endogenous matter. It is not because of endogenous factors that the epithelium of the skin becomes differentiated; it is compelled to differentiate by its surface situation. That cells have, besides, a certain innate disposition to differentiate cannot be denied; it manifests itself in explants very strikingly. The essence of the matter is that healing beneath the dried crust represents an effect of the outside world via the crust so that — directly or through the crust — exogenous factors are at play. I do not, therefore, regard Dr. Tsanev's experiment as decisive; the renewal of crust in our experiments seems to me of greater significance. Prof. Tokin alone concurred with me in the opinion that dedifferentiation did not mean a process in which a given cell — I am now exaggerating — became first keratinized and then, under some influence, again dedifferentiated. That this cannot be the case is shown by many signs. One is dealing here with a constant exchange of cell populations, a process that is exceedingly rapid in cases of wound healing. Both proliferation and exchange occur at a rapid rate. To Dr. Lehoczky I must reply that it is almost impossible to experimentally provoke abnormal regeneration. It is strange that we are unable to create experimental conditions in which the skin would imitate the abnormal regeneration of the portional epithelium. This is why human skin, under certain pathological conditions, is an especially suitable object. It cannot be reproduced in animals because cauterization produces a surface crust and thus the normal process. Inflammation, on the other hand, produces a quite abnormal milieu, and phenomena occurring therein cannot be used for inferences concerning the normal process of wound healing. We tried to study the process beneath various ointments, and found that crust had formed under them. The crust was different inasmuch as it was saturated with fat, but the process itself remained the same. I must agree with Prof. Romhányi who regards the analogy between tumorous infiltration and the infiltration occurring in the process of wound healing as far fetched. He referred to the escape of cells from cellular contact as a problem of importance. The fact that cancer cells perform amoeboid movements, and epithelial cells move freely in explants has abundantly been treated in the literature. Properly speaking they, too, emigrate like amoebas. Explants are, therefore, unsuitable for studying the spreading of epithelial cells, whereas we have no other method of examination. Dr. Csaba wanted to know whether and how far the necrotized mass of the crust and the tumour were analogous. The greatest part of the tumorous mass consists of epithelium, and the rest is composed of wandering cells, connective tissue and other matter, while epithelium is negligible in crusts which contain chiefly wandering cells, vessels and connective tissue. The two structures are, thus, fundamentally different, while both are constantly exchanged. Their similarity is nevertheless striking and biologically important. The decomposition product contains a substance which stimulates the epithelium; this is very important, has already been alluded to in one of the comments, and will surely be discussed by Dr. Tsanev. The supposition in question has been constantly recurring since 1894 in connection with regeneration, and diverse substances (peptides; products forming during the decomposition of nucleic acid; etc.) have been suggested in this respect.

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COMPARATIVE STUDIES ON THE TONOFIBRILLAR STRUCTURE IN THE COURSE OF WOUND HEALING AND SKIN CARCINOGENESIS

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Synopsis

Comparative studies have been made to examine the intraepithelial fibre system of the epithelial papillae formed during the regeneration of the epidermis and carcinogenesis. In connection with regeneration there is a well developed tonofibrillar structure in the epidermis from the very beginning. In the course of skin carcinogenesis there is a clearly recognizable tonofibrillar structure, which is roughened, hypertrophic at sites, in the hyperplastic epithelium, in the papilloma and even in the differentiated keratinizing squamous cell cancer. The tonofibrillar substance and the amount of alpha-keratin are decreased in the undifferentiated, reticularly growing squamous cell cancer. Throughout the process of regeneration the epithelium remains highly differentiated. In the undifferentiated squamous cell carcinoma the disappearance of the inter- and intracellular fibrillar structure is presumably associated with a decrease in the forces keeping the tumour cells together, in the forces of cell contact.

Introduction

In recent years, light and electron microscopic investigations have brought us nearer to an elucidation of the finer structure and to some extent of the function of the intraepidermal fibre structures in health and disease [9, 15, 20, 21, 22]. Many workers have studied the problems of the so-called cell contact, of the interactions between cells [2, 3, 4, 23].

By electron microscopic studies it was shown that the tonofibrils arise through condensation of the elementary tonofilaments. By means of the intercellular bridges on the cell membrane the neighbouring cells are in contact, though tonofibrils do not always pass through the bridges. The so-called dermoepidermal junction, the connections between the basal membrane and the epithelial cells, is ensured by a similar filamentar structure.

The tonofibrils can be readily studied by means of the polarisation microscope [10, 14, 16, 18, 19]. Their double refraction is due to the presence of keratin material. The intraepidermal fibrillar structure has an important role to play in keratinization. The X-ray diffraction studies [1, 5, 6, 8, 17] have shown that the tonofibrils contain alpha-keratin, which is converted to beta-keratin in the stratum corneum. Alpha-keratin is digested by pepsin and trypsin.

Comparative studies on the tonofibrillar structure in the course of wound healing and skin carcinogenesis may supply valuable information, because

1. the appearance and disappearance of tonofibrils inform us as to the grade of differentiation of the cell, the measure and nature of cornification, and

2. the morphological changes may permit a deeper insight into the changes in cell contact.

Materials and methods

The materials and methods used in this study were described in the previous paper [12]. The histological investigations have been supplemented by a polarisation optical method. For this purpose the sections were stained with gallocyanin-chrome alum, because in the sections so stained the double refraction of the tonofibrils is enhanced. Thus, the dye produces dichroism shown by polarisation microscopy, and facilitates at the same time histological and cytological orientation. The birefringence was determined by means of a Köhler compensator. To demonstrate alpha-keratin, frozen sections were digested with trypsin dissolved in 0.01 per cent phosphate buffer (pH 7.73) and the times when the tonofibrillar double refraction disappeared and when isotropia appeared, were recorded.

Some sections were treated by the phenol method of Ebner, after which the collagen fibres showed negative double refraction along their longitudinal axis, while the tonofibrils retain their longitudinal positive double refraction, and thus, the two kinds of fibre can be differentiated easier from one another. The anisotropic fibres showed a white pattern, with black lines perpendicular to them.

Wound healing

In the figures we can see side by side the photographs taken in white light and polarized light of the various cutaneous changes.

In the intact skin of the rat a well developed intraepithelial fibrous structure is visible, showing positive double refraction along its longitudinal axis, under it, in the dermis fibres showing negative longitudinal birefringence can be recognized as a result of the phenol reaction (Fig. 1 *B*).

The fundamental property of the epithelium growing under the crust is that the epidermal fibrillar structure is perfectly developed from the start (Fig. 1 *D*). Such a well-developed, and at sites even hypertrophic tonofibrillar structure can also be found there where the strata of epithelial cells growing forward show broom-like expansions or club-like thickening (Fig. 1 *F*), and the tonofibrils even remain in the cells which in this way remain deeper. With the onset of cornification the tonofibrils become more marked, rougher in the epithelium growing under the crust. On the other hand, when the epithelium becomes overdifferentiated, *i.e.* it shows marked cornification, the tonofibrillar structure melts into a cornifying mass. When wound healing has come to an end, the epidermis covering the surface has an intact fibrillar structure similar to that visible in the control specimen (Fig. 1 *H*).

Skin carcinogenesis and cancer

At the site of application the cancerogenic hydrocarbons initiate a hyperplasia of the epithelium, followed by the development of papilloma, then by that of cancer. For a while the tumour shows organotypic growth resembling the original structure of the epithelium. At that time it is composed of differentiated cells. With the onset of infiltrative growth its structure changes, becoming similar to a reticular network, the cells being undifferentiated. The figures show how the intraepithelial fibrillar structure changes while the epithelium undergoes this transformation.

In the hyperplastic squamous epithelium and in the papilloma every cell contains tonofibrils (Fig. 2 *A*). Also the intercellular bridges, otherwise not visualized in the mouse's skin, become visible. At sites we can see frag-

mentation of the thickened tonofibrils. The differentiated cornified squamous epithelial cancer possesses a well developed epidermal fibre network (Fig. 2 *D*), with the cornification well marked. In this phase fragmentation of the thickened

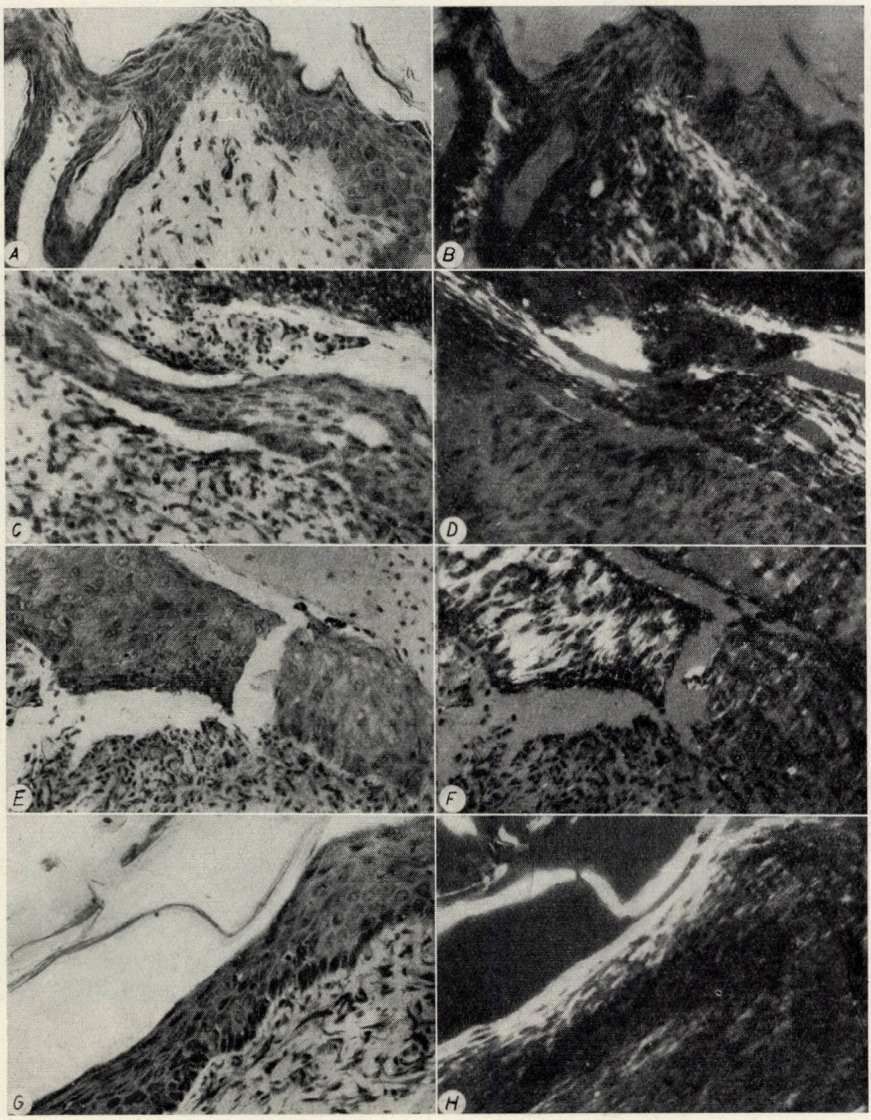

Fig. 1 *A*, *B* Intact rat skin, with well-developed tonofibrillar structure. The tonofibrils show positive birefringence along the longitudinal axis. The birefringence of the fibres in the dermis is longitudinally negative, as a result of the phenol reaction. *C*, *D* Regenerating epithelium growing under the crust having a well-developed epidermal fibre network. (12 days after inflicting wound). *E*, *F* On the 14th day of wound healing. The epidermal papilla (club) growing under the crust, with hypertrophic tonofibrils in some areas. *G*, *H* On the 21st day. The epidermis of the healed wound surface, with tonofibrillar structure of regular appearance. Gallocyanin-chrome alum stain. ×200

Fig. 2 *A, B* Epidermis of mouse, with hyperplasia following staining with methylcholanthrene. Every cell contains tonofibrils, which are hypertrophic at sites, thickened and fragmented. *C, D* Tonofibrillar structure similar to that seen in the previous figure in differentiated keratinizing squamous cell cancer. *E, F* Undifferentiated area of experimental cancer of the skin. Tonofibrils occur in very small numbers, those present show slight birefringence. Gallocyanin-chrome alum stain. ×200

tonofibrils is even more marked. On the other hand, in the anaplastic cancer showing infiltrative growth and not capable of differentiation we hardly find any tonofibrils; even those present are of irregular shape and show slight birefringence.

Since the alpha-keratin of the tonofibrils is readily digested by trypsin, we have tried to determine the amount of alpha-keratin or the quantity of tonofibrils in the above described structures by means of trypsin digestion. We have determined, therefore, the time required by trypsin to lyse the tonofibrils in areas differentiated in different degrees. In the undifferentiated cancer

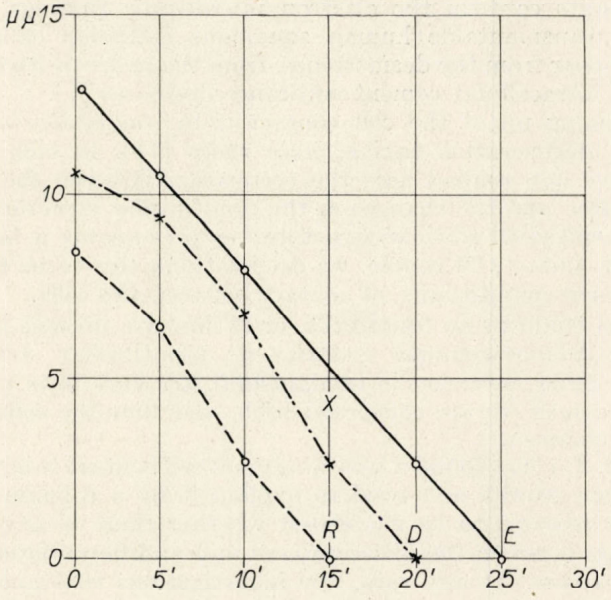

Fig. 3. Trypsin digestion curves of the tonofibrils of mouse skin treated with methylcholanthrene. Lysis for 25 minutes in the hyperplastic epithelium (curve *E*), 20 minutes in the differentiated cancer (curve *D*), 15 minutes in the undifferentiated, reticularly growing cancer (curve *R*)

the slight amount of tonofibrillar structure still present becomes lysed and isotropic in a matter of 15 minutes (Fig. 3). In the differentiated cancer complete lysis takes place in 20 minutes, in the hyperplastic cancer in 25 minutes. All these results prove that with the advance of carcinogenesis the amount of alpha-keratin and together with it the quantity of tonofibrils decrease in the epithelial structure.

Discussion

The studies of tonofibrillar structure permit the assessment that there is a similarity between regeneration and the onset of carcinomatous transformation. The epithelium which has become hyperplastic as a result of treatment with carcinogenic hydrocarbons, the papilloma formed and the differentiated cancer, as well as the epithelium produced in the course of regeneration, possess a well-developed intraepithelial fibrillar structure, The analogy is the more conspicuous, since during these processes the epithelium shows an increased tendency to differentiation (keratinization). Similar results were

obtained by other methods in studies on the tonofibrillar structures of the hyperplastic epithelium [13].

In the course of anaplasia the quantity of the fibrillar structure gradually decreases and hardly any or no tonofibrils can be found in the undifferentiated, infiltrating, then reticularly growing epithelium showing carcinomatous transformation.

These results confirm the electron microscopic findings, according to which in the transplantable human squamous epithelial cancer the tonofilaments disappear from the desmosomes, from the nodes of Bizzozero and the amount of the intracellular cement substance decreases [7].

The loosening up of the cell contact is in connection with the "unicellular" fatty degeneration taking place there [11]. In lack of a suitable method we have not studied here the correlation between the "unicellular" fatty degeneration and the changes in the tonofibrillar structure.

The intraepithelial fibrillar structure has presumably a fairly large role in securing cell contact. Of course, we do not think this to be the sole factor responsible for the maintenance of contact between the cells.

Basalioma contains no tonofibrils, or if they are present in it, are rudimentary [14]. Adenocarcinoma contains no tonofibrillar structure at all. These tumours differ, however, in fundamental structure from the keratinized squamous cell cancer we are concerned with, and thus the cells are freed by some other mechanism.

Coman [2, 3, 4] has found a loosening up of cell contact to be characteristic in carcinomatous growth and tried to explain it by a calcium deficiency of cancer cells. It remains to be elucidated whether there is any difference in calcium contents between the differentiated and undifferentiated parts of the carcinomatous tissue. At any rate, our investigations substantiate the view that in the undifferentiated parts the cancer cells undergo disjunction easier, which makes it possible that such cells be carried away to an increased measure. Thereby it can be understood why the cells carried in large numbers can settle more often at other sites.

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Contributory Discussion

Prof. Romhányi: It is interesting that it is easy to recognize (partly quantitatively) a submicroscopic character in this cell association which means a further step toward the determination of dedifferentiation. The tonofibrillar system in the cells of the squamous epithelium is fairly primitive so that it is not easy to tell whether, in processes of regeneration, say, in an area of inflamed granulation tissue, one is faced with squamous epithelial cells. Differences of resistance between more mature and less mature cell associations, as demonstrated by means of digestion tests, are highly interesting. How and to what extent does the gallocyanin method strengthen the birefringence of the tonofibrils, and how could we utilize it for the demonstration of squamous epithelial cells?

Dr. Sugár: It was by mere chance that we stained tonofibrils with gallocyanin. The dye enhanced the birefringence fibrillar structures $2^1/_2$- to 3-fold, and so I was able to demonstrate the existence of structure even in the basal layer. The same phenomenon was visible under the electron microscope as well.

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DATA TO THE HISTOCHEMISTRY AND BIOCHEMISTRY OF ULCUS CRURIS (VARICOSE ULCER)

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Synopsis

The authors report on the results of the examinations made in tissue specimens excised from 16 patients with crural ulcer. It is suggested that a circulatory disturbance is in the centre of the pathogenesis of crural ulcer which leads to hypoxaemia and oxygen deficiency in the tissues. The histological pictures show the known pattern of distended, congested capillaries. It has been found that, as compared to that of the normal skin, the lactic acid content of the skin of patients with crural ulcer is increased 2- to 3-fold. A significant increase has also been found in the biochemically determined mucopolysaccharide content of the skin-tissue. The latter could be demonstrated histochemically, too, inasmuch as acid and neutral mucopolysaccharides could be disclosed to be present in significant quantities.

The development of the ulcer, its poor tendency to healing, the impairment of circulation, the histological and histochemical changes, the high lactic acid concentration and the increase of mucopolysaccharides unequivocally indicate that the metabolism of the skin has shifted from the oxybiotic (having been in the foreground earlier) toward the glycolytic, fermentative metabolism. This shift is considered to be a biological adaptation of the tissue.

Crural ulcer (ulcus cruris) takes a special position among the cutaneous ulcers of different origin. The special pathological position is to be ascribed, on the one hand, to the fact that the pathomechanism of the disease is complex and not clarified yet in all its details, and, on the other hand, to the resistance of the condition to the various methods of therapy. These problems are extensively discussed in surgical and dermatological papers. Recently, the problems of the pathogenesis and treatment of crural ulcer have been dealt with, in considerable detail, at a joint meeting of the Hungarian Surgical Society and the Society of Hungarian Dermatologists, on March 21, 1959 [14]. While in recent years the views concerning the pathogenesis have been brought on the whole to a common denominator, yet no final conclusions could be drawn concerning therapy.

It may be surmised that in the centre of the pathogenesis of crural ulcer there is an impairment of peripheral circulation, which, however, is influenced in turn by several factors, such as heredity, occupation, gravidity, a tendency to thrombosis, etc. [14, 22, 29, 13]. It has been proved in many papers that besides the venous changes of different nature and severity (phlebothrombosis, thrombophlebitis, varicosity, valvular defect, etc.) morphological or functional changes could also be demonstrated in the arteries: arterial hypertension [15] was demonstrable in about 50 per cent of all cases, and in 14 per cent of ours, as factors influencing crural ulcer. Relying chiefly on the evidence put forward earlier by French authors, the role of the capillaries was stressed on grounds

of histological studies by Szodoray and Sóvári [23] and on the basis of capillary microscopic examinations by Bugár-Mészáros [3]. Morphological changes in the nerves of the ulcer tissue were demonstrated first by Szodoray and Sóvári, then by Huriez. In view of the latter findings the team of Rajka and Bugár-Mészáros [19] has suggested that such conditions be called neuroangiosis cruris haemosiderica, and the same team headed by Máramarosi [15] suggested the term ulcus cruris neuroangiosum to denote the ulcers which develop in connexion with them. Bugár-Mészáros found that in 70 per cent of the crural ulcer cases the capillaries of the skin showed diminished resistance.

The above outlined morphological and functional changes, in the three parts of the vascular system, make it understandable that as a result of the impaired circulation in the skin of the leg an oxygen deficiency (anoxia, Huriez) develops in the tissues and according to one of the authors [8, 9] the metabolism of the skin shifts in the direction of anaerobic glycolytic metabolism. This may presumably account for the accumulation of mucopolysaccharides in the cutaneous tissue of the ulcerated leg, that can be demonstrated by suitable chemical and histochemical techniques. The increase of mucopolysaccharides leads to the induration, sclerosis of connective tissue in the skin surrounding the ulcer.

To prove the validity of this hypothesis, chemical, histochemical and histological studies were made in 16 patients with crural ulcer, partly to substantiate the anaerobic glycolytic processes, partly to prove that mucopolysaccharides were, in fact, present to increased amounts in the tissues. It is emphasized that the examined ulcers had been present for at least one month, but mostly they had persisted for several months.

Lactic acid in tissues was determined by Barker and Summerson's method as well as by the technique of Friedman, Cotonio and Schaffer, as modified by Tankó. Hexosamine in blood was estimated by Rimington's method or by the technique of Morgan and Elson [17], as modified by Szabolcs and Tankó [21]. The essence of the latter modification is that the determination is made almost from beginning to end in the same vessel and thereby the limits of error are reduced to below 5 per cent. Hexosamine in tissues was determined by the method of Boas. The above biochemical determination have been made by Éva H. Oláh, and Mária B. László, biochemists.

To substantiate the *conception* that in the affected skin metabolism would shift from the earlier, mainly oxybiotic toward the anaerobic glycolytic processes, the clinical and histochemical studies have been supplemented by two kinds of biochemical determination as well as by metabolic studies: (1) The lactic acid content of the skin was determined in 16 cases. (2) The mucopolysaccharide content of skin tissue was measured in 12 cases (see Table 1).

The lactic acid concentrations found in the 11 patients involved in this study were: 184, 189, 95, 168, 147, 174, 121, 113, 123, 128 and 194 mg per cent, representing a mean value of 148.8 mg per cent. Together with the mean reported for 5 cases earlier by Krompecher and co-workers [8, 9] (mean: 129 mg per cent), we get a mean value for the total of 16 cases of *142.6 mg per cent.*

Let us compare this value with the normal values for the skin: 56, 58, 48, 60, 81, 37, 45, 32 and 48 mg per cent, representing a mean value of *51.6 mg per cent.*

Although most of the values listed above had been obtained in various parts of the body, the 45 and 32 mg per cent ones were obtained in the normal skin of the leg and the one of 48 mg per cent in the skin of the knee region, and for this reason they can be used as controls in the present material. Thus, if

Table 1

Results of the tests made in 16 cases of ulcus cruris

No.	Date	Name	Age years	Hexosamine content, mg% in		Lactic acid in skin, mg%	Lactic acid production (anaerobic) in 1 hour, mg%
				serum	skin		
	1960						
1.	July 13.	J. K.	49	106.6	1047.5	184.1	—
2.	July 15.	Mrs. O. J.	41	90.0	—	—	—
3.	July 15.	Mrs. Zs. Gy.		82.0	856.9	—	—
4.	July 16.	Cs. F.	75	83.3	—	189.2	—
5.	Sep. 27.	Mrs. V. S.	56	109.3	515.1	94.6	—
6.	Oct. 8.	Mrs. N. G.	58	79.3	361.0	168.2	—
7.	Oct. 13.	D. J.	72	90.7	430.0	147.1	—
8.	Oct. 22.	Mrs. L. J.	58	116.3	403.6	174.5	—
9.	Oct. 26.	Mrs. Sz. K.	31	86.3	511.6	—	—
10.	Nov. 1.	Mrs. B. G.	64	96.3	375.0	—	—
11.	Nov. 11.	Mrs. B. L.	46	75.0	445.8	121.3	—
12.	Dec. 14.	Mrs. Sz. J.	61	109.7	599.0	113.0	—
	1961						
13.	Jan. 3.	Mrs. Sz. S.	40	88.0	—	—	99.1
14.	Jan. 6.	K. M.	66	—	512.4	122.8	68.5
15.	Jan. 12.	Mrs. B. I.	54	97.7	—	128.0	162.2
16.	Jan. 27.	S. J.	61	96.7	610.4	193.7	176.9

we compare the 51.6 mg per cent normal value with the mean lactic acid contents of 142.6 mg per cent found in the skin around the crural ulcer, it can be stated that the skin in crural ulcer contains nearly three times as much of lactic acid as the normal skin. The development of the ulcer, its poor tendency to healing, the impairment of circulation, the capillary pathology visible in the histological specimens, in the cases of longer duration the functional and morphological anomalies of the capillaries, the hyalinisation of the connective tissue, as well as the histochemical (increase of mucopolysaccharides) and biochemical (high lactic acid values in all 16 cases) findings unequivocally indicate that oxybiotic metabolism is interfered with. It is a biological law that under such circumstances the tissue metabolism switches over to fermentative processes, supplying about 10 times less energy [25, 26]. In four of our cases we examined the lactic acid production in 1 hour of tissue specimens taken from the area of the ulcer, under anaerobic conditions, in the Warburg apparatus. The results obtained were: In the case of Sz. S. the quantity of lactic acid produced in 1 hour under anaerobic conditions, as related to that found at 0 hour, was 99.1 mg per cent, in the case of K. M. it was 68.5 mg per cent, in the case of B. I. 162.2 mg per cent, and in the case of S. J. it was 176.9 mg per cent. The mean value is thus 126.7 mg per cent. It is to be pointed out that the tissue lactic acid concentration was high even at 0 hour in each

of these cases (see Table 1). From this follows that the tissue around the ulcer is capable of producing considerable amounts of lactic acid under artificially created anaerobic conditions. The above findings tend to indicate that with the oxybiotic metabolism impaired (as is suggested by the data mentioned above) the fermentative processes of the Embden–Meyerhof scheme might have gained relative preponderance. Those outlined above supply data and arguments to prove that just like in tuberculosis [4] and cancer [24, 25, 12, 18, 16, 28, 2, 9, 10], in ulcus cruris, too, metabolism may have shifted in the direction of anaerobic glycolytic fermentation.

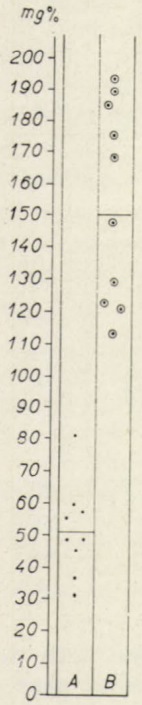

Fig. 1. Comparison of the lactic acid contents of the normal skin (A) and crural ulcer skin (B)

This hypothesis is also confirmed by another series of biochemical tests. In 12 cases we also determined the hexosamine contents of the ulcerated skin and obtained a mean value of 555.7 mg per cent (Table 1). This value, as compared to the mean tissue hexosamine contents of the human organism, is extremely high, because according to the investigations of Krompecher, Oláh and László the normal hexosamine content of the different human organs averages 400 mg per cent, and we have found a mean value of 239.7 mg per cent in three specimens of normal human skin (263.7, 190.9, 264.6 mg per cent). As compared to these values, the one of 555.7 mg per cent represents a significant increase.

The increase of the hexosamine content of the tissues is another evidence supporting the hypothesis that in crural ulcer the metabolism of the skin shifts from the oxybiotic toward the anaerobic glycolytic, fermentative processes.

In addition we have tested 15 ulcer patients for serum hexosamine by the Morgan–Elson method, as modified by Szabolcs and Tankó [21] (Table 1). In 5 the serum hexosamine level was normal, in 5 it was moderately elevated, in 4 it was about as high or higher than the levels usually found in myxoedema. The serum hexosamine level was slightly lower in but one of the 15 patients tested. These findings permit further evaluation.

Each biochemically tested case was studied histologically, too. To demonstrate mucopolysaccharides, the sections were stained partly by the cresyl violet and toluidine blue metachromatic staining method, and partly by the combined Hale and PAS technique of Ritter–Oleson. In general, the preparations showed strong PAS positivity in the wall of the capillary bunches and interfibrillary Hale positivity in the loose connective tissue around them (Fig. 2). In some cases the Hale positivity was very strong, in accordance with the high tissue hexosamine contents found by biochemical methods. We have known, for a long time that mucopolysaccharides are increased in the processes of wound healing. In his monograph on wound healing Washburn [27] points out that in the course of wound healing certain groups of cells are forced to anaerobic metabolism.

In some of our cases of crural ulcer of longer standing and showing connective tissue sclerosis the Hale positivity was less marked and the PAS

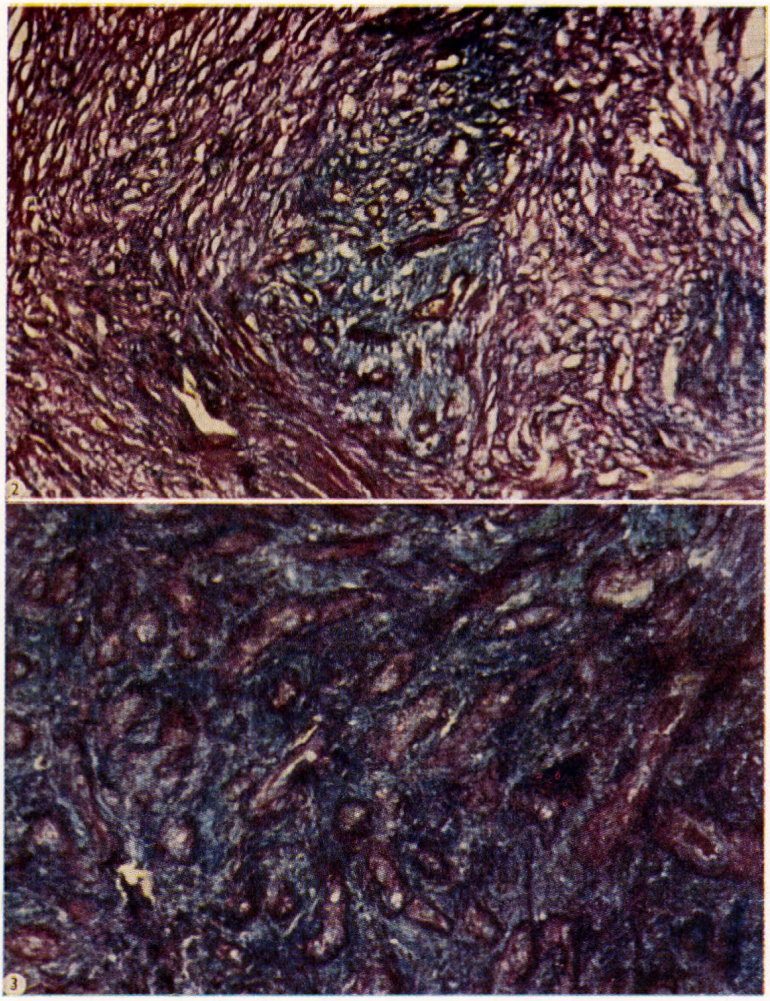

Fig. 2. In the basal skin tissue around the ulcer the acid mucopolysaccharides (Hale positive, blue) have increased. The capillaries are dilated, have thickened walls. In some areas the capillaries have perished, and the connective tissue ground substance contains PAS positive substance (red). Ritter—Oleson staining

Fig. 3. Dilated capillaries with thickened wall from the neighbourhood of an ulcer. The basal tissue is strongly Hale positive. The capillary walls show PAS positivity. Ritter—Oleson staining

reaction of connective tissue fibres was in the foreground (Fig. 3). The latter observation indicates that mucopolysaccharides showing Hale positivity and cresyl violet metachromasia observable at first interfibrillarly are built in the collagen fibres later. In some graver cases a greater accumulation of PAS-positive material was found. Among histoenzymological reactions the strong tetrazolium reaction in the connective tissue of the ulcer, as well as the uneven phosphatase activity in the dystrophic capillary walls were conspicuous (Fig. 4). On the basis of observations outlined above following statements can be made.

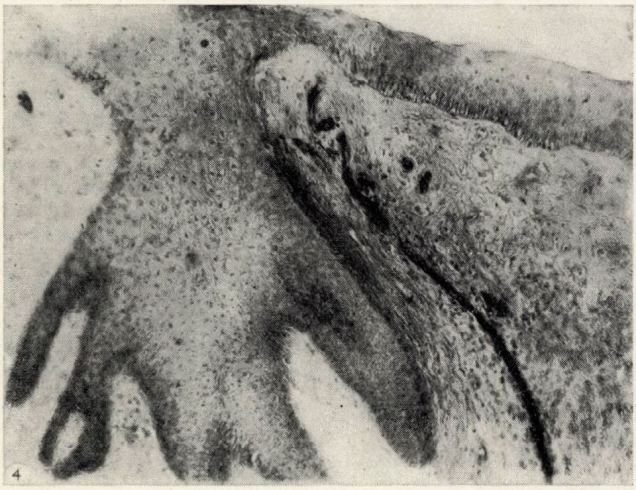

Fig. 4. Phosphatase activity of the capillaries in crural ulcer tissue (Gomori's method). Only the arterial ramus of the capillary shows positivity

In individuals with crural (varicose) ulcer such histochemical and biochemical changes can be demonstrated in the skin around the ulcer, as are indicative of definite alterations in the metabolism of the crural skin. In crural ulcer the skin contains 2 to 3 times as much lactic acid as the normal skin. The tissue mucopolysaccharide content of the skin, too, was definitely increased in crural ulcer, while the blood hexosamine level of the crural patients showed no unequivocal behaviour (the levels were partly normal, partly elevated). Histochemical studies showed mostly an increase of the Hale positive interfibrillary substance in the connective tissue around the ulcer and a PAS positivity of the pathologically altered capillary walls. On the basis of all these data it may be surmised that in the skin adjacent to the crural ulcer the metabolism has shifted in the direction of anaerobic glycolysis, resulting in an increase of the mucopolysaccharides, which in turn gives rise to sclerosis of the connective tissue later.

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Contributory Discussion

Dr. SZÁNTÓ: The number of reports is increasing according to which chronic arteriovenous shunts are in the background of ulcus cruris. I suspect that some of the structures described as capillaries in the reports are actually arteriovenous shunts. I share the view that polychromasia, the accumulation of mucoproteins, is really the morphological manifestation of hypoxic conditions. There exist data to show that polychromasia represents a necessary and regular phase of wound healing. This hypoxic phase is perhaps necessary for the regeneration of connective tissues. It becomes pathologic if the process stops at the stage of hypoxia and precisely this may occur in cases of ulcus cruris. There is a great amount of polychromatic (*i.e.* mucoprotein-containing) tissues in embryos, and it is known that the blood contains considerably less oxygen before than after birth. That the connective tissue of embryos is jelly-like may be connected with these chronically hypoxic conditions. This leads to problems of embryogeny and ontogeny, even to phylogeny: conditions in this respect ought to be studied in poikilothermic lower vertebrates whose heart is not four-chambered and in which the level of oxygen is low.

Dr. SZŐNYI: Static measurement of accumulation of a certain intermediary product is not suitable for deciding the question as to whether metabolism is glycolytic or oxidative in a given area. Lactic acid, the final product of glycolysis, is intermediary *in vivo*. It is oxidized under normal conditions. If a static measurement shows increase in the amount of lactic acid in a case of ulcus cruris, it may simply mean that its transport is obstructed and need not indicate increased glycolytic activity. Production of lactic acid should be followed routinely in clinical practice.

Dr. PEER: We have been told that widely distended capillaries are visible in cases of ulcus cruris. I understood that the nervous apparatus shows certain pathologic changes at the same time. The situation is similar in respect of pediculated skin transplants: while the measurements of skin-temperature give satisfactory results and the blood supply of the transplant appears to be good, innervation — especially as regards sensory nerves — is most deficient, almost lacking. Such transplants are highly susceptible to ulceration if exposed to stresses; ulcers so formed are similar to ulcus cruris. We have, as a matter of fact, designed experiments in which the nerves of the transplanted flap are brought into contact with the corresponding sensory nerves of the host area.

Our attempts to facilitate trophic and sensory innervation in this manner have unfortunately failed. It is, nevertheless, presumable that innervation is the main problem in cases of both ulcus cruris and trophic ulcer. Investigations in this respect should be started in connection with ulcus cruris; the ninhydrin test is very sensitive and has proved satisfactory for a study of dermal innervation.

Dr. SUGÁR: X-ray ulcer is undoubtedly a local process, and a comparative study of X-ray ulcer and ulcus cruris might yield interesting results.

Prof. SZODORAY: Almost all cases of ulcus cruris were subjected to angiography with Prof. Ladányi. In no cases were signs pointing to the existence of shunts observed on the radiographs.

Dr. SZÁNTÓ: According to the literature the shunts are at the precapillary level; hence they are not to be seen on radiographs. The problem should be approached using other methods.

Prof. SZODORAY: Mucopolysaccharides accumulate even during acute regeneration. A powerful accumulation of this kind is characteristic of ulcus cruris. Although the assimilation of mucopolysaccharides may encounter difficulties, one has to deal here with chronic hypoxia characteristic of ulcus cruris. We shall run the experiment suggested by Dr. Szőnyi who divided the excised piece and inspected one fragment instantly, the other after a one-hour incubation. The problem of capillaries and nerves is extremely difficult. Dr. Bugár-Mészáros, collaborating with us, studied the capillary pattern in remote areas, *e.g.* on the lip and the conjunctiva. He repeatedly encountered Miller's dystrophic capillary picture, a sign of generalized acrocyanosis. Congenital or hereditary dystrophy or dystonia may be the cause of abnormal capillary arrangement all over the body. Entire families have been known to be acrocyanotic. Several Soviet authors have attempted to treat ulcus cruris by means of neuropharmaca, but they obtained no satisfactory results. Though there exist analogies between the X-ray ulcer and the ulcus cruris, their histological manifestations are different. I think I could distinguish the one from the other if I saw them together in a preparation. In X-ray ulcers there are no capillary tufts in the new vessel walls, while the connective tissue reveals signs of hyaline degeneration and obliteration. Let me touch upon another problem: we speak of brady-

trophia and sluggish metabolism, while dehydrogenase activity is increased. We ought to investigate this matter.

Dr. NIWELINSKI: May I ask if the enzyme you studied was succinic dehydrogenase? Was any correlation between the intensity of the reaction and the clinical characteristics of the case? And which tissue was the most active?

Prof. SZODORAY: We used the tetrazolium reaction.

The reaction was always positive in every case, but no correlation of the staining with the clinical picture was observed.

The most intense staining was found at the fundus of the ulcus, in the so-called fibroblast layer underneath the crust and in the growing epithelium on both sides.

Prof. KROMPECHER: There are two sharply distinguishable types of hypoxia. If the milder form of hypoxia affects granulation or connective tissue capable of differentiation, acid mucopolysaccharides, hyaluronic acid, chondroitinsulphuric acid, etc. will be formed. If the severe form of hypoxia acts upon a non-plastic tissue, it degenerates, as can be observed in cases of ulcus cruris. These data are now being published in detail. We examine our material not only chemically but analyse it histologically, histochemically and biochemically as well. With my collaborators (Hadházi, Oláh) we have performed such analyses on cartilage, in tuberculous granulomas and in cases of ulcus cruris. Chondrogenesis by way of neodifferentiation was accompanied by a reduction of capillary cross-sections, a decrease in the haemoglobin level and an increase in the amount of mucopolysaccharides. My collaborators made the Warburg test in more than 250 dogs, but the results showed great deviation. It was, therefore, not possible to obtain Warburg values. The patients do not like large-scale excisions, but we shall attempt it. Only a combination of various methods yields reliable results. This applies to the study of X-ray ulcers, too.

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REGIONAL DIFFERENCES (AXIAL GRADIENTS) IN WOUND HEALING

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Synopsis

The wound healing rate, respiration, glycolysis, temperature and histological structure of various skin areas were studied. The increased healing rate of cranial wounds compared with caudal ones appears not to be due solely to circulatory causes as was accepted until recently. This phenomenon exists not only in humans but also in the shoulder and gluteal areas of quadrupedal mammals with a horizontal back (albino rat, guinea pig, dog) and in fish. The latter is merely observable by histological methods. There are similar gradients of skin temperature in man and guinea pig. The difference in wound healing rates is simultaneous with that of endogenous respiration and anaerobic glycolysis of rat skin. The phenomena are assumed to be in correlation with gradient theories concerning primitive organisms.

Introduction

An experience probably known for many centuries in surgical practice is, that wounds in different parts of the body do not heal equally well and at equal rate. Obviously this can be observed the most distinctly in superficial skin wounds and skin defects, as circumstances are far more complicated in injuries affecting the deeper tissues also, therefore, here the differences are less striking. Thus, it is known by every surgeon and mentioned in every surgical textbook, that wounds of the head and face heal well and in a relatively short time, rarely suppurating.

Attempts have been made for some time to explain these phenomena. Good wound healing on the head and face is explained by good blood supply; whereas poor wound healing and ulceration observed rather frequently on wounds of the leg and feet is generally interpreted as the consequence of the vertical human posture. Because of this, venous return in the lower extremities is hindered as circulation must be maintained against the hydrostatic pressure of a large liquid column. This is also an undoubted fact. The different circulatory factors gave a probably well founded explanation in both cases, this is probably the cause why this interpretation is accepted without much further investigation and doubt.

We feel that surgeons are generally less well versed in experimental biology than the experts in this field, and this fact might have contributed to our accepting the above explanation as largely satisfactory.

In our experimental studies to be reported here, we tried at first to clarify whether this explanation holds, or whether there are also other factors involved in the different healing rates of various body regions.

Experimental biology has numerous data indicating that in many lower animals gradual differences exist along certain somatic axes, considering certain reactions, such as the extent and rate of regeneration, metabolic activity, sensitivity towards noxious agents and chemicals. We do not wish to review the rather extensive literature of this complicated question here, the less so, because the biologists present are certainly far better acquainted with this subject, and therefore we are only referring to the fact that Child's [4, 5] gradient theory was based on these facts. When referring to Child's disputed theory we do not wish to commit ourselves in any way. From Hungarian authors we mention the studies of Török [28] and those of Dévényi and Kellner [8] concerning quantitative evaluation of wound healing.

The above results of experimental biology indicate — in short and simple wording — that in certain animals regenerative potential shows a more or less gradual decrease from the cranial to the sacral end. A similar decrease was found in metabolic activity and other reactions.

Considering these facts, our original question was put as follows. Are the observed regional differences in wound healing of human beings entirely due to the circulatory conditions mentioned, or besides these are there some biological factors involved which might be analogous to the axial gradients observed in experimental biology. In other words, whether there are in addition to the factors mentioned more fundamental biological phenomena involved in human wound healing.

To approach this problem it had first to be determined whether higher animals, particularly the quadrupedal mammals with a horizontal posture, show differences of wound healing similar to those in humans.

Methods

During the first phase of our work standard wounds were made along various cranio-caudal axes on three kinds of mammals (albino rat, guinea pig and dog) and the progress of healing as well as the time of complete healing was recorded. Respiration and anaerobic glycolysis of skin excised from the same sites was determined by Warburg's method. Histological structure of the same skin was also examined.

The temperature of skin in various body regions of humans and guinea pigs was determined.

We attempted to investigate the course of healing in fish, using the same technique as on mammals, to gain data on a non-mammal vertebrate as to the existence of the regularity found in mammals.

The influence of the animal's age on the experimental results was investigated.

The above metabolic studies were extended to the changes of regenerating tissue during wound healing.

The asymmetry found in our recent experiments raised the problem of (left — right) laterality, of unilateral dominance.

Experimental procedure

400 albino rats, 55 guinea pigs and 10 dogs were used for the experiments. The animals were of different age and sex. Sex and weight was recorded, but the animals were used indiscriminately because the postulated regularity — it existent — was supposed to be independent of age and sex. Rats weighed between 140—250 grams, guinea pigs between 300 and 800 grams. A few animals were above or below these values. In the experiments designed to determine the possible influence of age, rats were used. The

weight of the young animals ranged from 61 to 77 grams, that of adult rats from 114 to 143 grams. Dog experiments were carried out on bastards of various age and sex.

Generally two wounds were made, only in some experimental series were 4 wounds inflicted on the same animal. 10 animals were generally used in each series. To achieve comparable wound of uniform size, the wounds were made with the same trepan. The diameter of the trepan used on rats and guinea pigs was 10.5 mm, that used on dogs of 25 mm. In every case wound was made through all skin layers to the underlying fascia, and the skin was cut around and was removed by a few scissor cuts in the connective tissue without damaging the muscular fascia. We tried to eliminate any possible error arising from the unequal stretching of the skin by using a simple decive, a metal ring fixing the skin concentrically by its milled surface. The trepan was applied through the central aperture of the ring (Fig. 1).

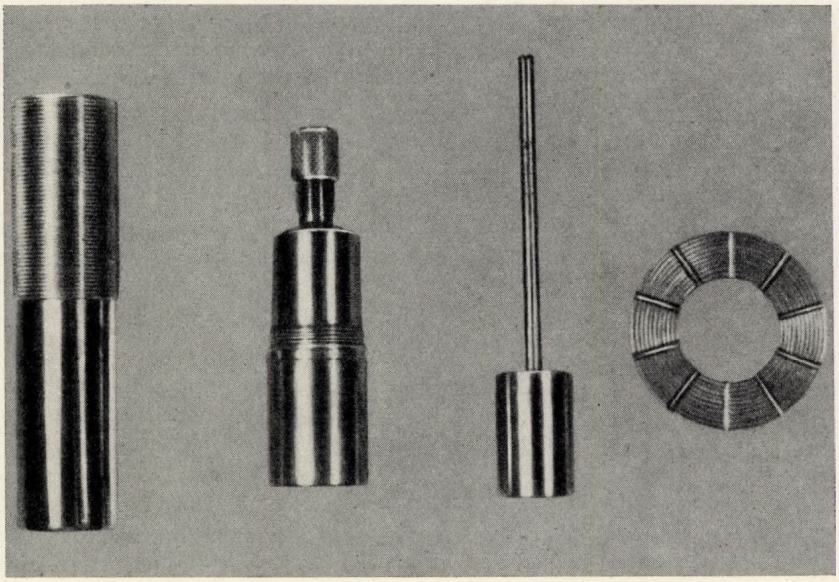


Fig. 1. Trepan used for wounding with the ring constructed by us to fix the skin

Standard wounds were made on the dorsal surface along two cranio-caudal, one ventral, and one dorsoventral axis. One axis ran through the shoulder and gluteal region, placing the wounds on sites well underpadded by muscle, *i.e.* the skin areas over the deltoid and gluteal muscle, respectively. The other axis ran through the middle line of the back with wounds on the nape and above the sacrum. The ventral axis was formed by wounds placed in the middle line, on the cranial part of the chest and the middle of the lower abdomen. The two dorso-ventral axes were along the nape and the centre of the upper part of the shest, and above the sacrum and the middle line of the lower abdomen. In one group (10 animals) the healing of wounds made on the caudal and cranial parts of the abdomen were compared.

The course of healing was followed up by placing cellophane on the wound and tracing the edges, according to Carrel and Du Noüy [3], without planimetric measurements. The scab covering the wound was not removed, as this would have influenced the healing.

Tracing the margin on cellophane placed on the wound is not suitable for the precise recording of wound size, because the line thickness and inaccuracy of the hand are both sources of error. The margin of error is $+10\%$. Since in the present experiments but gross differences were to be evaluated this technique of preliminary experiments proved to be rather satisfactory.

We are aware of the fact that the line of the wound margin recorded without the removal of the scab does not represent the exact wound size, the less so, because healing progrediates under the scab. Since in the present experiment the subject to be investigated was not the absolute wound size itself but the relative healing rates of the two wounds, as the source of error was present at the two wounds equally it could be neglected on comparison. Our aim was not to compare the wound sizes at any given time, but to compare the times of the complete healing of the two wounds. Also for this reason we omitted the planimetry. The wounds were considered to be healed when a scab was not observable any longer and the wound seemed entirely epithelized. It is quite probable also in this case, that epithelization was already complete for some time before losing the scab, but this inaccuracy was also present in both wounds, and thus negligible in comparing healing terms, without affecting statistical evaluation.

After determining the suitable intervals for recording wound, respectively, scar sizes by the above method in preliminary experiments, drawings were made on the day of wounding, on the 3rd, 5th, 7th, 9th, 11th day and from then on daily. The cellophane drawings were stuck on a table in chronological order, with the drawings made at the same time arranged one below the other in sequence synoptical tables of the healing of simultaneously inflicted injuries.

The evaluation of experiments was carried out by reading these tables.

Histological examinations were performed after formalin fixation with haematoxyline-eosine and Van-Gieson staining.

Skin temperatures were determined by using electric skin thermometers (type Ugodi and Biotherm II) on 14 healthy individuals and 9 guinea pigs, the animals were previously depilated at the necessary points. Determinations were made on 36 different sites on the surface of the human body, always measuring identical points. On guinea pigs 6 points of the surface were measured.

Wound healing

Cranio-caudal axes

Mammals

As briefly reported previously in each of the three kinds of mammals, altogether in about 130 animals, it was conclusively demonstrated that the more cranially placed wounds showed a healing rate significantly higher than those placed more caudally. The differences between the results of experiments conducted at different seasons were negligible.

As it may be seen in Table 1, in guinea pigs the shoulder wounds in average healed in 14.5—15 days, the wounds in the gluteal area in the average in 17,6—18,2 days. Thus, the difference between the wound healing of the two regions was 3.1—3.2 days. The healing time of the more caudal wound was by 22% longer than that of the cranial one.

As shown in the table, similar results were seen in rats.

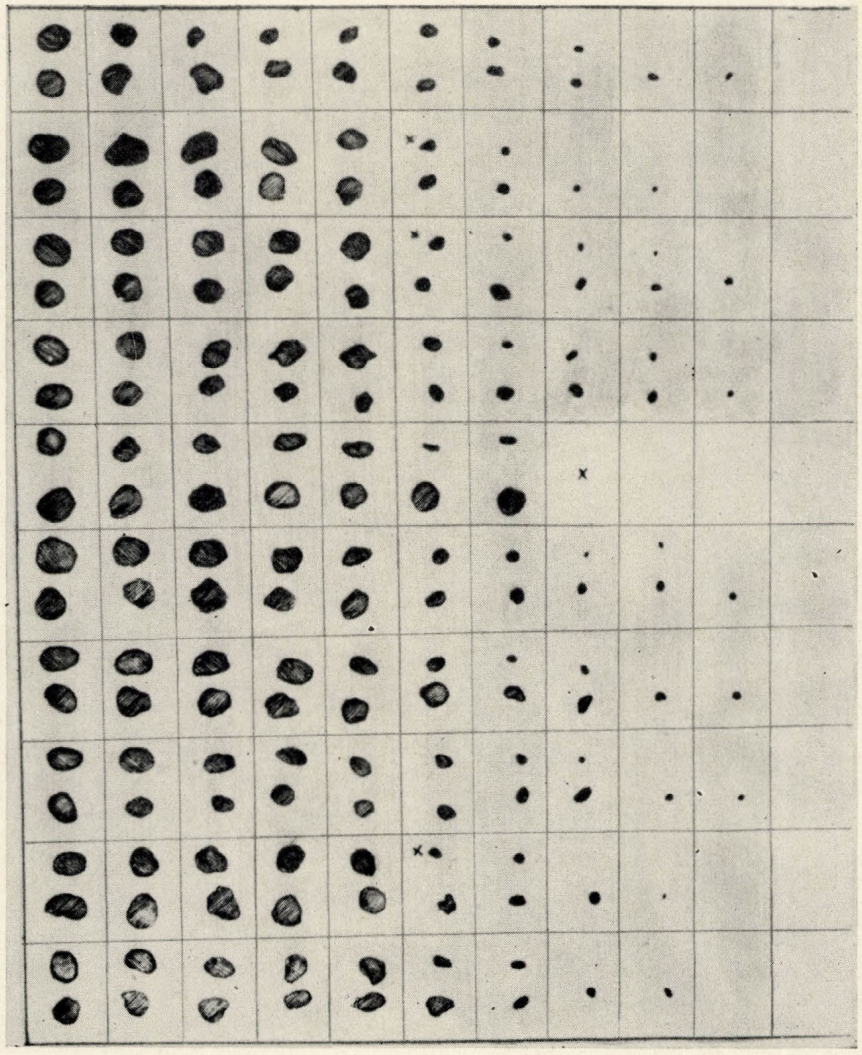
Wound healing in dogs took a longer time, but it must be remembered, that, though absolutely larger wounds were made on dogs (25 mm trepan diameter) relative to the body surface, these were smaller than in rats. Average healing time of caudal wounds was 26.6 days, that of the ones sited more cranially 33.1 days, the difference amounting thus to 6.5 days. 127% of the healing time of the anterior wound was necessary for the healing of the posterior wound (shoulder, rump).

The difference of the healing term between wounds made on the nape and sacrum exceeded the difference between shoulder and rump wounds in both rats and guinea pigs. 3.7 to 5.1 days was the average difference found

Table 1

Cellophane table showing the registration of wound healing progress

Experimental series No 1 Guinea pig. Date of wounding 6.11.1955. Trepan diameter 10.5 mm. Depth of wounding: skin + subcutaneous connective tissue. Average weight of animals 210 g

No.	Day of wounding: 2.	3.	5.	7.	9.	11.	13.	15.	17.	19.		difference in days	Note
1											anterior wound posterior wound	+4	
2											anterior wound posterior wound	+3	anterior scab detached
3											anterior wound posterior wound	+2	anterior scab detached
4											anterior wound posterior wound	+2	
5											anterior wound posterior wound	—	animal lost by intercurrent illness
6											anterior wound posterior wound	+2	
7											anterior wound posterior wound	+4	
8											anterior wound posterior wound	+4	
9											anterior wound posterior wound	+4	anterior scab detached
10											anterior wound posterior wound	+4	

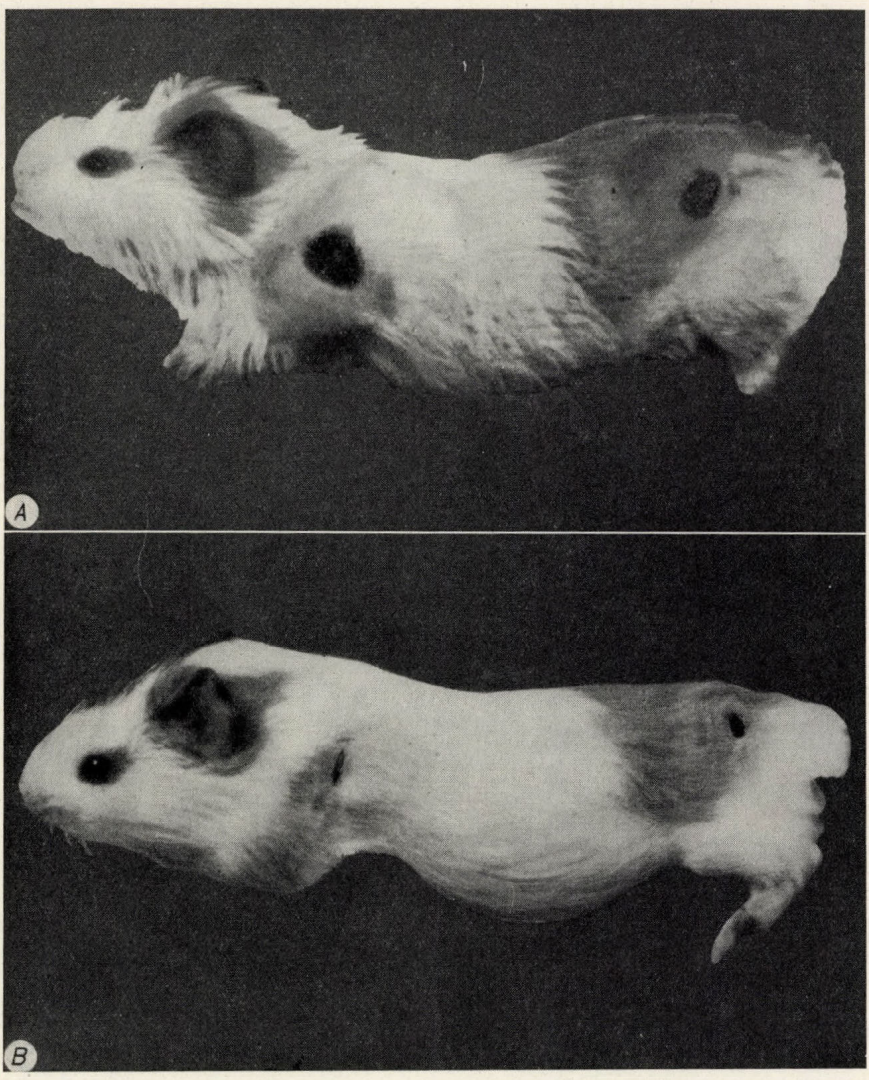


Fig. 2 A — Guinea pig on the day of wounding. *B* — The same animal 10 days later

in rats and 4.6 to 7.0 days in guinea pigs. The healing time of caudal wounds was 25 to 58% longer than that of cranial wounds along this axis. The difference was highly significant.

Seperate experiments were carried out to study the influence of age on the healing rates and the axial gradients. The experiments on dogs had already given some informative data, *i.e.* among the animals there are three young ones about one year old, from the same litter. Their wounds healed more rapidly, and the difference in healing time between their frontal and caudal wounds was greater than in the older animals used in the same series. Their cranial wounds healed within 21 days, whereas in the others it took 25—39 days.

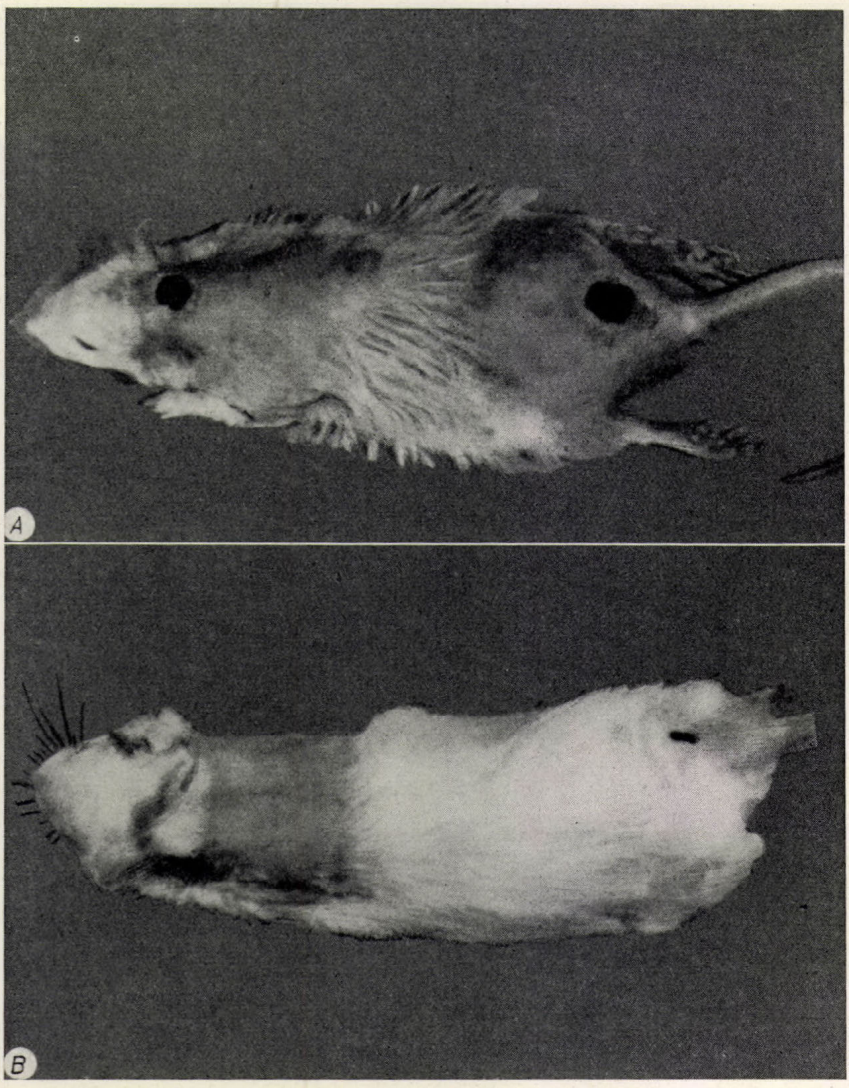


Fig. 3 A — Albino rat on the day of wounding. *B* — The same animal 10 days later

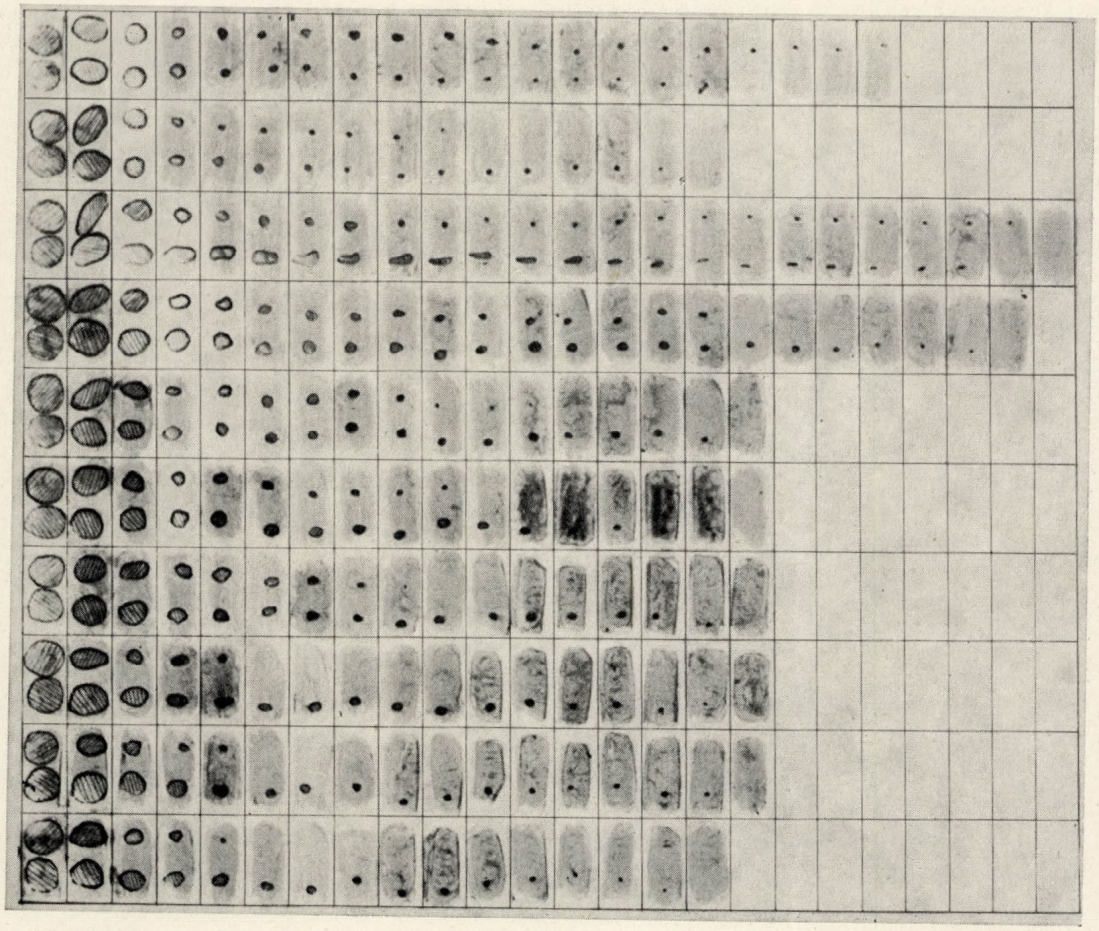
In these three animals the difference between the healing times of frontal and caudal wound was 10 to 11 days, whereas in the others it was 4 to 7 days. (Table 2, animals number 8, 9 and 10.)

A seperate group was investigated to study the effect of age. In ten young albino rats, weighing between 61 and 77 grams, the time required for complete healing averaged 12.2 days on the nape, 16.2 days in the sacral wound (Table 3). In 10 adult, but not old rats (Table 4), weighing 114 to 143 grams wounded at the same time, the time required to heal averaged 15.8 on the nape, 19.5 days over the sacrum.

Table 2

Registration of the progress of wound healing in dog

Experimental series No. 5. Date of wounding: 9.1.1956. Trepan diameter 25 mm. Depth of wounding: skin + subcutaneous connective tissue



No.	Difference in days	Note
1	— 4	anterior wound suppurated
2	+ 5	
3	— 1	Old scar next to anterior wound
4	+ 6	
5	+ 4	
6	+ 6	
7	+ 7	
8	+11	
9	+11	
10	+10	

Table 3

Progress of wound healing in young albino rats

Date of wounding: 21.6.1956. Trepan diameter 10.5 mm. Depth of wounding: skin + subcutaneous connective tissue. Average weight of animals 67.4 g

No.	Weight of animal g	Sex	Day of wounding	5.	9.	10.	11.	12.	13.	14.	15.	16.	17.	18.	19.	20.	Difference in days	Note
1	62	♀															+5	
2	76	♂															+6	
3	67	♂															+1	
4	61	♂															+5	
5	66	♂															+4	
6	76	♂															+3	
7	77	♂															+2	
8	67	♀															+3	
9	63	♀															+2	
10	59	♂															+9	

Table 4

Progress of wound healing in adult albino rats

Date of wounding: 21.6.1956. Trepan diameter 10.5 mm. Depth of wounding: skin + subcutaneous connective tissue. Average weight of animals 124.3 g

No.	Weight of animal g	Sex	Day of wounding	5.	10.	11.	12.	13.	14.	15.	16.	17.	18.	19.	20.	21.	22.	23.	Difference in days	Note
1	117	♀																	+3	
2	123	♂																	+1	
3	141	♀																	+2	
4	120	♀																	+7	
5	143	♂																	+5	
6	114	♀																	—2	Abscess formation on site of anterior wound
7	120	♂																	+2	
8	125	♀																	+5	
9	124	♀																	+6	
10	116	♀																	+3	

Table 5

Regional differences in wound healing

Experimental series	Site of wound	Number of evaluated cases (n)	Average healing time in days		Healing time difference in days	Average*	t	P %	Significance	Date of experiment	Note
Guinea pig											
I.	shoulder-rump	9	15.0	18.2	3.2	122	8.805	$\ll 0.1$	most highly significant	November	
IV.	shoulder-rump	10	14.5	17.6	3.1	122	6.118			December	
Albino rat											
II.	shoulder-rump	7	13.0	16.7	3.7	128	2.120	< 0.1	most highly significant	November	
III.	shoulder-rump	10	14.1	15.5	1.4	110	3.373	< 1.0	highly significant	December	
Dog V.	shoulder-rump	9	26.6	33.1	6.5	127	4.758	< 1	highly significant	January	
Albino rat											
VI.	nape-sacrum	7	13.7	18.8	5.1	137	7.253			February	Young rat
VIII.	nape-sacrum	10	12.2	16.2	4.0	133	5.440	$\ll 0.1$	most highly significant	June	Adult rat
IX.	nape-sacrum	9	15.8	19.5	3.7	125	5.30			June	
Guinea pig											
VII.	nape-sacrum	10	11.8	16.4	4.6	140	10.048		most highly	February	4 woundings
X.	nape-sacrum	9	12.1	19.1	7.0	158	7.27	$\ll 0.1$	significant	July	Control
X/a.	nape-sacrum	4	12.0	19.0	7.0	158	5.30			July	
Guinea pig											
X.	abdomen-chest	9	10.1	11.8	1.7	117	3.06	< 2	dubious	July	4 woundings
X.	chest-nape	9	11.8	12.1	0.3	103	0.54	> 60	none	July	on each
X.	abdomen-sacrum	9	10.1	19.1	9.0	191	8.78	$\ll 0.1$	most highly significant	July	animal
Guinea pig											
XI.	lower abdomen-epigastrium	10	10.4	11.5	1.1	112	2.65	< 5	marginal case	August	

* Healing time of more slowly healing wound expressed in % of the ones healing faster.

Another group (guinea pigs) served to study the wound healing differences along the dorsoventral axes. Further in some series it was to be examined whether our findings on the dorsal surface (wound healing axial gradients) applied also ventrally. Inflicting more injuries to the animals than previously (abdomen, chest, nape, sacrum) raised the question of a possible influence of multiple woundings on the healing process. Therefore, control animals were simultaneously wounded on the nape and sacrum only. The nape wound healed averegely in 12, the sacral one in 19 days, in these control animals. In the animals with four wounds the nape wound healed in 12.1 the sacral one in 19.1 days on an average. Their abdominal wound healed on an average in 10.1, their chest wound in 11.8 days. The healing time differences between wounds of various somatic areas was subjected to a statistical analysis. This

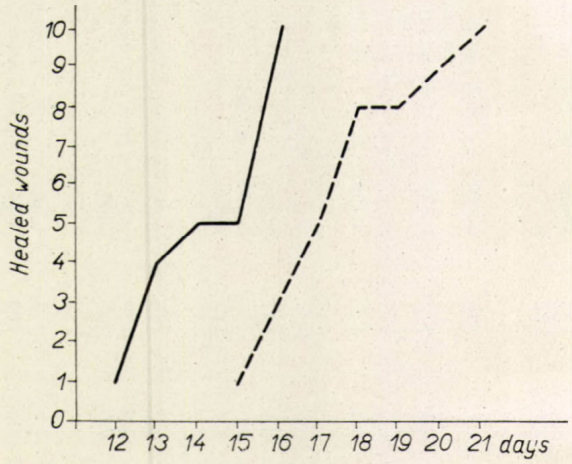


Fig. 4. Cumulative curve of wound healing. Guinea pig ——— shoulder – – – rump

treatment revealed that the difference between abdomen and sacrum is highly significant, however, the one between nape and chest is not significant (see Table 5, X and X/a) and that between chest and abdomen is doubtful.

The healing difference of wounds inflicted on the upper and lower parts of the abdomen were also studied on guinea pigs. Out of the 10 animals used in the experiment 4 showed no difference of healing time between wounds on the epigastrium and lower abdomen, in 6 cases a difference of 1 to 4 days was found, the wound on the lower abdomen healing in a shorter time. On statistical treatment this difference proved to be a marginal case from the point of significance (see Table 5, XI).

Attention was paid to the possible influence of sex and colour, *i.e.* albino or not albino animals throughout. Our experiments brought completely negative results in this direction, so that the conclusion must be drawn that these factors are not involved. Figs 2 and 3 illustrate these points. In Fig. 2 *A* a guinea pig is shown on the day of wounding, in Fig. 2 *B* the same animal 10 days later. The front wound getting smaller and healing within a shorter time may readily be seen. Fig. 3 *A* and 3 *B* illustrate similar changes, the former showing

Fig. 5 A — Mirror carp on the day of wounding, wounded along a lateral axis. The denominator in the marks on the figures for fish denotes the days after wounding. *B* — The same animal, 20 days after wounding. No diminution of wound observable

an albino rat on the day of wounding, and the latter the same animal 10 days later.

Table 4 shows the continuous diminishing of the wounds traced on cellophane. Inspection of the table shows the more cranial wounds — the upper ones in each row — becoming smaller and healing in a shorter time.

The difference of healing time between cranial and caudal wounds are clearly paraphed in the cumulative curves. In Fig. 4 the total number of

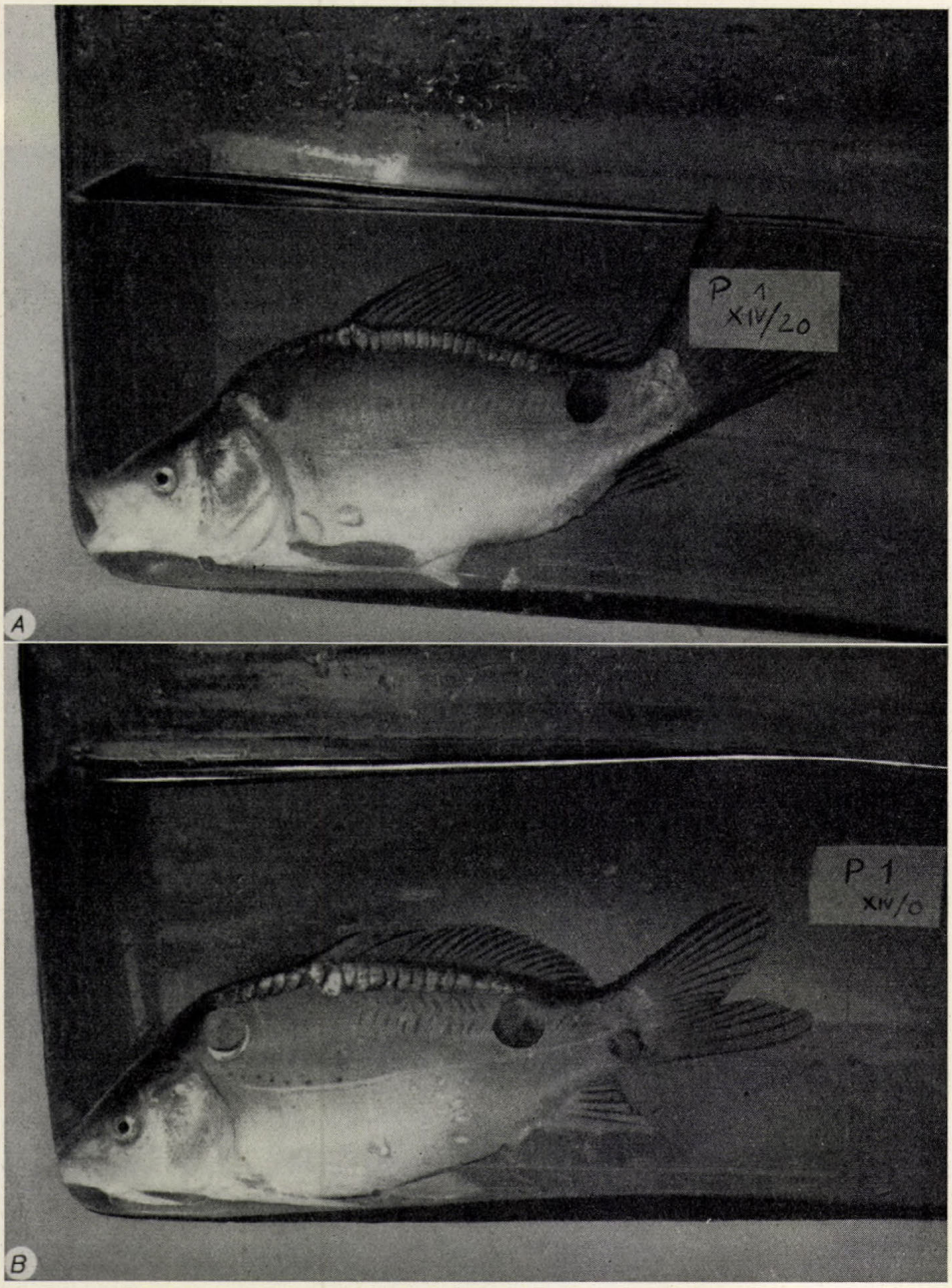


Fig. 6 *A* — Mirror carp on the day of wounding, wounded along a lateral axis. *B* — The same animal, 20 days after wounding. A slight diminishing of the frontal wound is observable

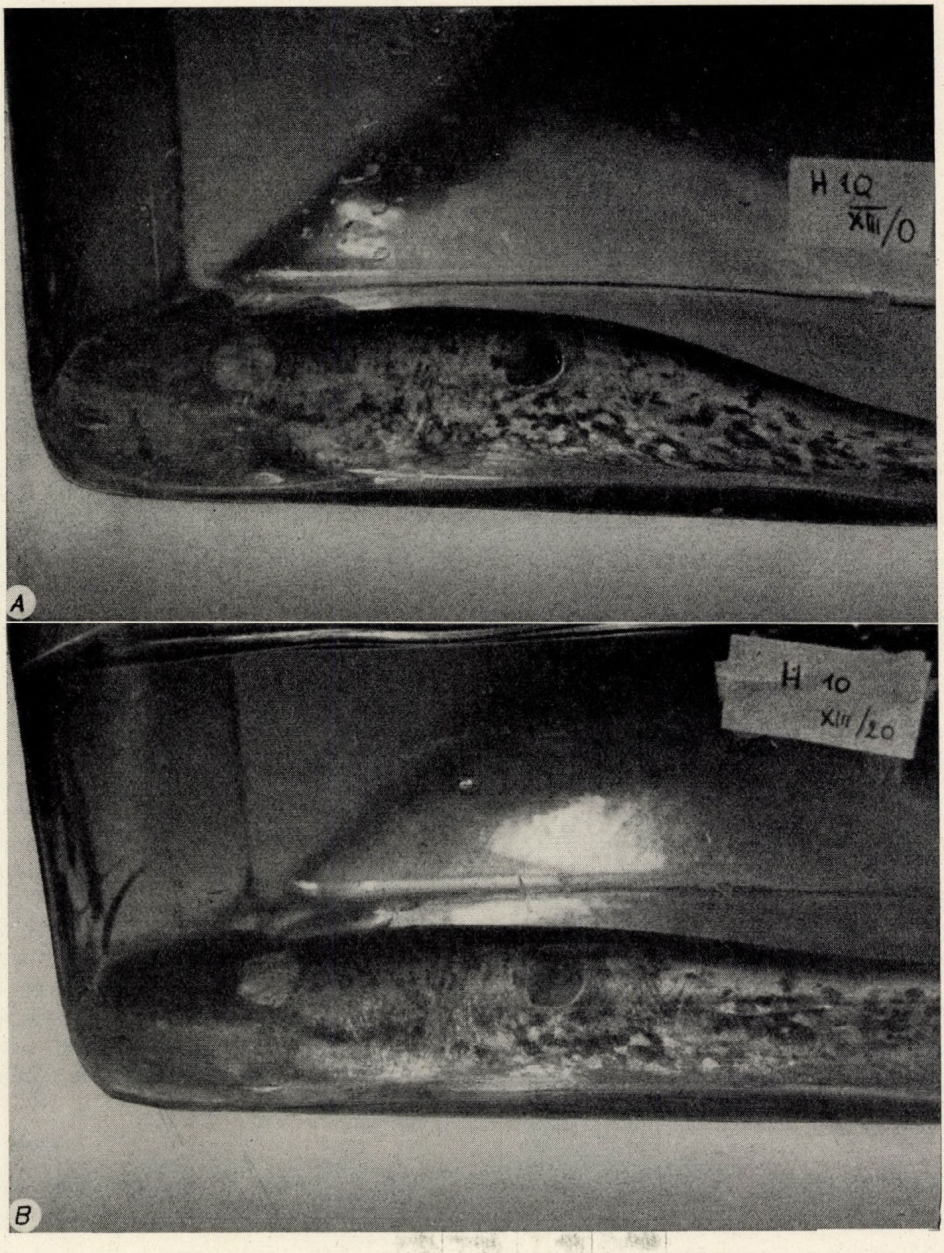


Fig. 7 *A* — Bullhead on the day of wounding, wounded along the central axis. *B* — The same animal 10 days later

Table 6

Skin temperature of the larger body regions in man

	Number of measurements	Average °C	Deviation
Lips	20	35.75	0.44
Head	30	35.71	0.43
Chest + abdomen	30	35.23	0.46
Back	20	34.96	0.41
Upper limb flexor surface	56	34.80	0.54
Upper limb extensor surface	57	33.76	0.69
Lower limb flexor surface	54	33.44	0.89
Lower limb extensor surface	76	33.09	1.33

completely healed wounds plotted against days of wounding. The retarded healing of the more cranial wounds, the later occurrence of the first healed caudal wound and the retardations of further healings compared to the more cranial ones, are readily to be noted.

Fish

As already mentioned when describing our methods, experiments were carried out on fish along the cranio-caudal axes, in the medial line of the back and the dorsal half of the body. Wounds were made by trepan according to our described method (Figs 5 *A—B* and 6 *A—B*).

As a result of the experiments we were led to concede that our routine technique which was efficient with mammals, was unsuited for registering the progress and establish the time of healing in fish. Wound healing in fish does not take place under a scab as in mammals, and a concentrical contraction does not occur. The wound is covered very quickly, within a few hours by a thin, grey, transparent layer which does not change appreciably for several weeks. Histological examination showed this sheet to consist of epithel. Rapid epithelization may have developed as a defence against the threat of osmotic damage from the surrounding water. We shall revert to this question when discussing the results of our histological investigations.

Table

Skin temperature of different body-regions

Number of animals	1.	2.	3.	4.	5.	6.	7.	8.	9.
Nape-sacrum	1.4	1.3	1.8	1.6	0.6	—0.2	0.2	0.8	1.2
Shoulder-rump	2.5	0.1	0.8	0.8	0.4	1.0	0.6	0.1	0.8
Fore — hind sole	6.8	4.3	0.2	1.5	0.5	—0.4	1.8	3.6	3.4

In some cases a tendency of more speedy diminishing of the more cranial wound surface was observed, however, not regularly (Fig. 6 *B*). The experiments were carried out on two kinds of fish, on mirror carp and catfish.

Skin temperature

Skin temperature in humans is the highest in the head, particularly on the lips and on the abdomen; it is lowest on the lower limbs, particularly on the dorsum of the foot and the sole. Guinea pigs show the same regularities as humans. Values are given in Tables 6 and 7, each data representing average values in °C of 14 humans, after reduction of measurements on different points of each area.

Skin temperature decreases in cranio-caudal direction in both humans and guinea pigs, the differences being markedly significant. There is no significant difference between abdomen, chest and back. It may be stated though, that the abdominal skin is warmer than the skin on the back, and its temperature approaches that measured on the head. This was found in humans as well as in guinea pigs.

Histological studies

Skin excised from the described areas was studied. The nape and sacral regions of 10 guinea pigs and rats, respectively, as well as the shoulder and gluteal areas of 4 guinea pigs and rats were examined microscopically after staining with hematoxylin-eosin and Van-Gieson (Figs 8 to 10). Part of this work had previously been published [26].

Summarizing our studies it may be concluded, that there is a marked difference in the histological structure of skin removed from different areas in both rats and guinea pigs. In both species it is equally characteristic that the epidermis on the nape is thick and multilayered consisting of voluminous cells. The hair follicles are rather long, rich in cells, with well defined inner and outer follicular membranes, their number also being higher than those on the more caudal parts. The loose subepithelial layer is less or even unobservable in more caudal areas, appearing on the skin of nape to be wide and cellular. The structure of the skin on the shoulder region is similar to that of the nape in both guinea pigs and rats, but the number and length of hair follicles as well as the dimension and cellularity of the subepithelial layer is distinctly smaller. Structure of sacral and gluteal regions in both kinds of animals differ

7

of guinea pigs and the differences in °C

Average	$\bar{x}$	s	t	P%	Significance
36.69—35.72	1.04	0.52	6.04	< 0.1	most highly significant
36.92—36.07	0.78	0.72	3.26	< 2	highly significant
32.99—30.58	2.41	2.33	3.11	< 2	highly significant

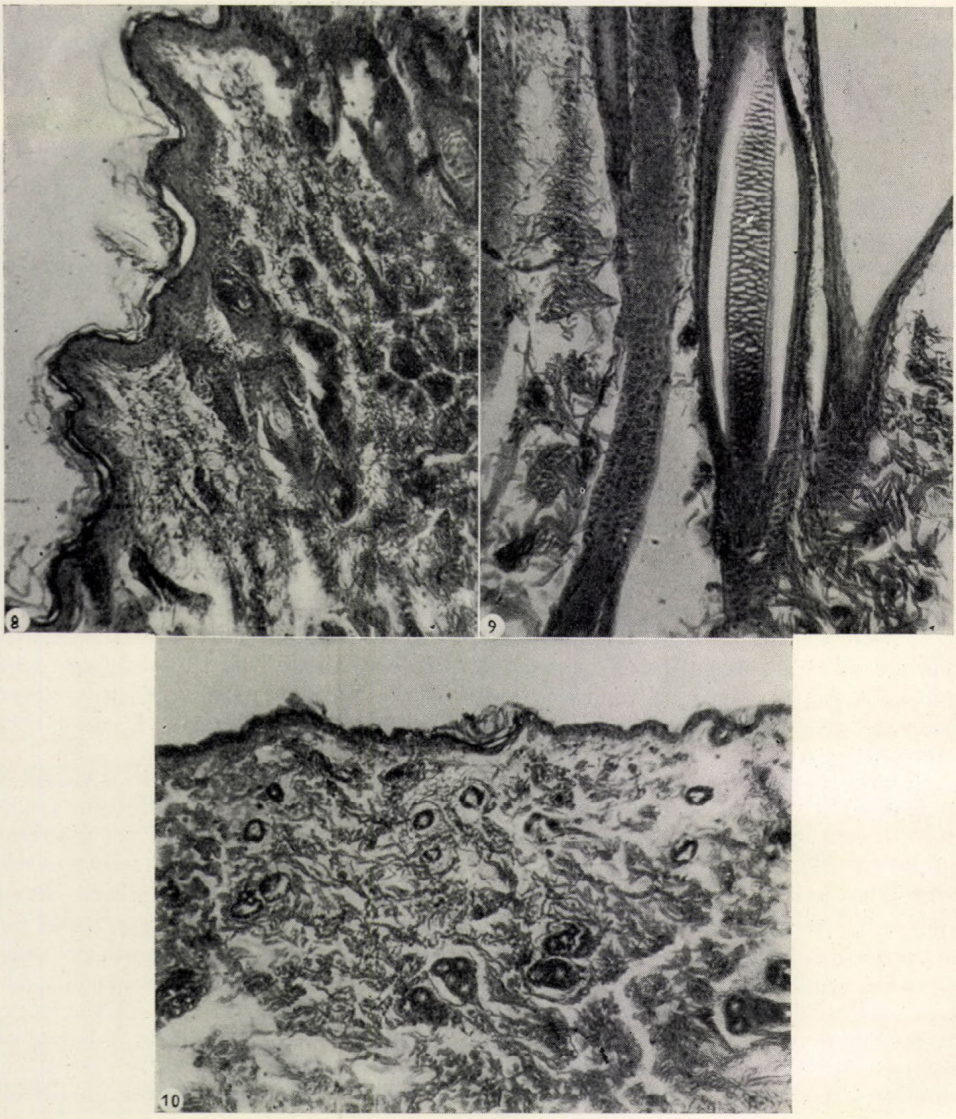


Fig. 8. Histological section of the nape skin of a guinea pig: broad epithelial layer with numerous voluminous hair follicles. *Fig. 9*. Nape skin of a guinea pig. High power. *Fig. 10*. Skin of sacral region of a guinea pig: thin epithelial layer, few hair follicles

markedly from that of the nape skin. Epidermis appears thinner, poor in cells, the number and size of hair follicles do not reach by far that of either nape or shoulder skin. The dermis is not divided into two layers but is uniformly acellular, consisting of coarse collagen fibres.

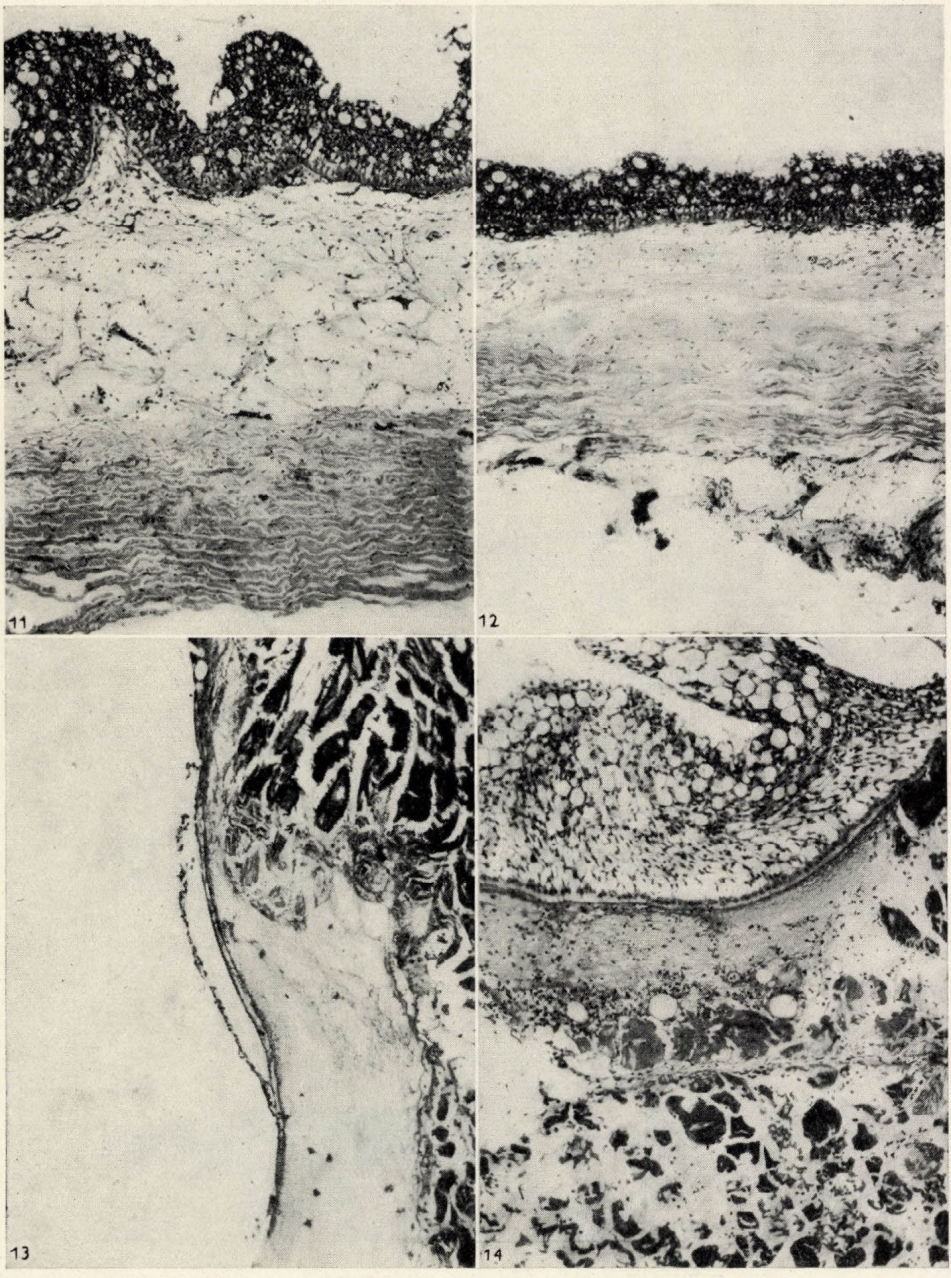


Fig. 11. Skin of a carp from the area near the head: broad epithel rich in muciparous cells, distinct papillarity. *Fig. 12.* Carp; skin of the caudal area. Thinner epithel, papillarity lacking. *Fig. 13.* Skin wound of a carp on the day after wounding. Thin epithelial sheet moving over the mucous coating. *Fig. 14.* Cranial skin wound of a carp 48 hours after wounding. Wound surface covered by broad, well differentiated epithel

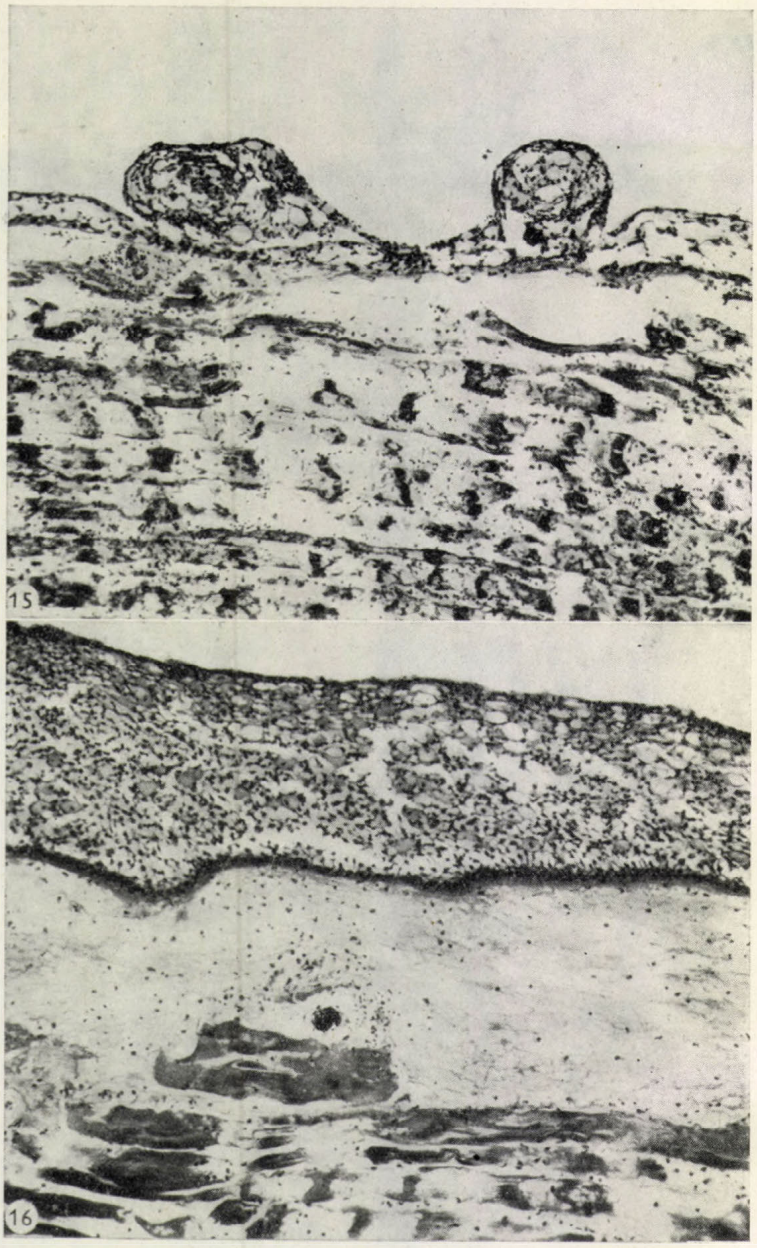


Fig. 15. Caudal skin wound of a carp 48 hours after wounding. Wound surface covered by thin, less differentiated epithel, occasionally forming fungiform regenerative centres.
Fig. 16. Cranial skin wound of a carp 4 days after wounding. Broad, well differentiated epithelial regenerate

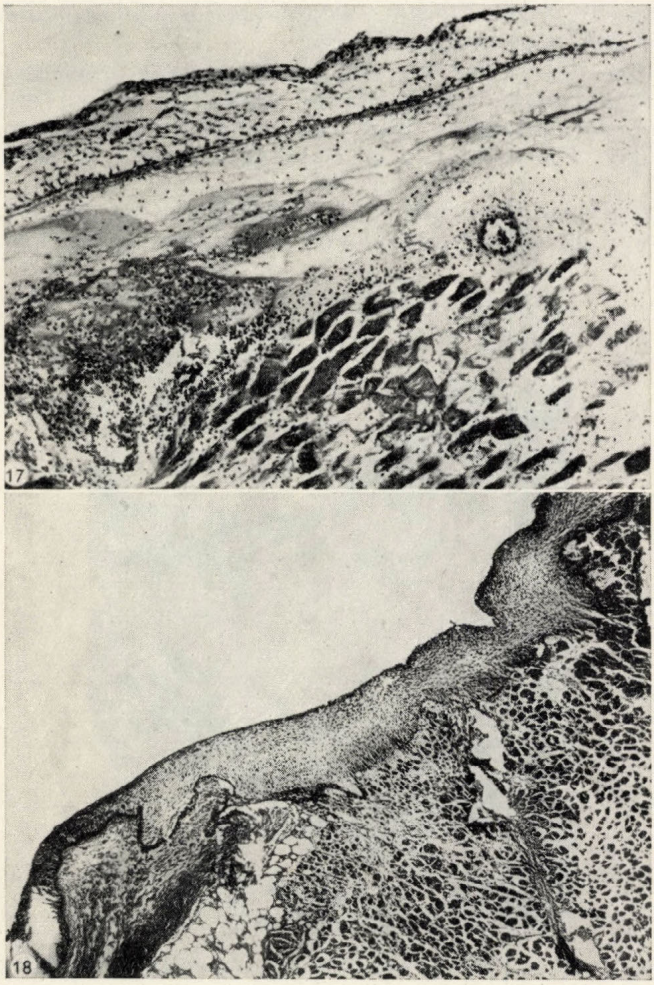


Fig. 17. Caudal skin wound of a carp 4 days after wounding. Epithel definitely thinner and less differentiated. *Fig. 18.* Carp skin wound, minimal subepithelial granulation and cicatrization between the musculature and the epithel. Epithel regenerate attached almost directly to the musculature

To summarize the histological results of our studies on carps, the following conclusions were arrived at:

In the skin of fish there are three distinct layers discernible.

1. Epithel
2. subepithelial loose connective tissue layer (with more or less adipose tissue)
3. dense, acellular connective tissue.

Beneath the latter follows directly, or separated by some loose connective tissue (occasionally adipose tissue) the musculature.

In the regions chosen for study (cranial and caudal skin areas) the structure of normal skin is definitely different.

On the cranial end there is a well developed loose connective tissue layer between the epithel and the dense fibrous connective tissue. Thick connective tissue papillae enter into the epithel, making the surface of the epithel occasionally papillar. The epithel layer is broad and rich in muciparous cells (Fig. 11).

On the caudal end the dense, fibrous connective tissue layer is of the same thickness or only slightly thinner than on the cranial end, the loose connective

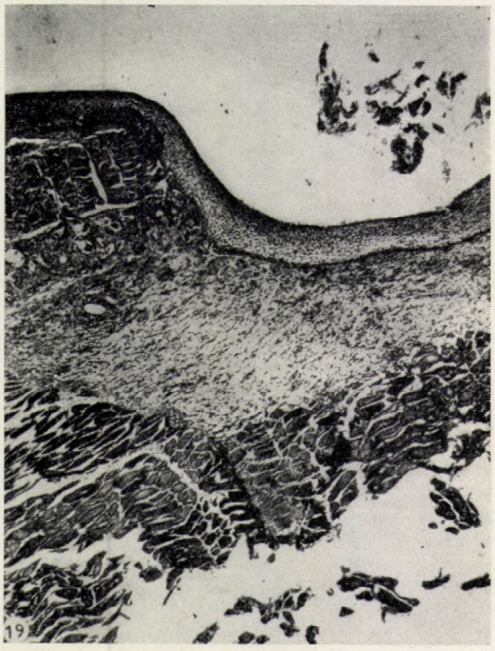


Fig. 19. Healing of skin wound in a carp after major epithelial injury. Between epithelial regenerate and the musculature there is a voluminous granulation tissue layer, respectively, cicatrization

tissue layer between this and the epithel is only poorly developed. The epithel appears thinner than on the cranial end, the connective tissue papillae are flatter and interdigitate less frequently. The number of muciparous cells in the epithel is smaller. A smaller number of melanocytes are to be found in the loose connective tissue than cranially. All this applies only to the animals studied approaching one year of age and only to the skin regions studied because of the well known fact that there are striking differences in layer thickness and the relationship of these to each other, the frequency of muciparous and pigment cells considering the abdominal and back skin, on one hand, and the age of animals, on the other (Fig. 12).

The wound inflicted by our standard size technique becomes completely epithelized within 48 hours. Wound surface becomes rapidly covered by the

secrete of the muciparous cells of intact epithel adjacent to the wound, and above this mucous film the epithel sheet moves very quickly across the whole wound surface. The study of this early epithel regenerate is technically rather difficult. Because of its loose adherence to its base it is easily destroyed even when handled with the utmost care (Fig. 13).

This makes the comparative study of the first day's regenerative processes impossible.

On the second day (48 hours after wounding) marked differences can be observed between the cranial and caudal end. Wound surface on the cranial end is covered by thick, well differentiated epithel, particularly near the wound margin (Fig. 14), whereas on the caudal end wound surface is covered only by a few rows of less differentiated epithel cells, occasionally forming fungiform regenerative centres (Fig. 15).

A distinct difference exists between cranial and caudal end also on the fourth day, epithel regeneration being far wider and more differentiated on the proximal wound than on the distal one. The numerous well differentiated muciparous cells are to be noted (Figs 16 and 17).

In later stages these differences between caudal and cranial regenerates quickly disappear.

A sharp line must be drawn in regeneration of skin wounds of fish, concerning the regeneration of epithel and subepithelial tissues.

In the regeneration of the latter no difference could be established when employing our method, because regeneration rate of subepithelial tissues varied with each wound. This deviation due to the various degrees of muscle injury on making the skin wounds, consequently the inflammatory granulation tissue reaction evoked for eliminating necrotic tissues was of various degrees. In case of minimal muscle injury the subepithelial granulation and cicatrization is minimal too, the epithel regenerate is almost directly attached to the muscles (Fig. 18). In case of major muscle injury, on the other hand, large granulation tissue layer and subsequent cicatrization develops between the epithel regenerate and the musculature (Fig. 19).

Tissue metabolism

Differences similar to the healing rates of wounds of various somatic areas were found, studying in vitro the energy producing metabolic processes of rat skin.

The following method was employed in collecting statistical data:

On the day preceding the experiments, the areas to be examined were depilated with a paste containing BaS. On the following day the samples excised by using a trepan, were quickly cooled in a Krebs—Ringer solution. After stripping off the fatty layer, samples were sliced, weighed and transferred to a Warburg flask, 100—200 mg tissue/flask. During a part of the experiments, this phase of work was carried out in a cold room [26]. In the oxygen uptake experiments Ringer phosphate solution modified by Krebs—Henseleit and adjusted to pH 7.4 was used with pure oxygen in the gas phase. Endogenous respiration was measured throughout, as preliminary experiments showed that added glucose as a substrate did not stimulate oxygen uptake. Krebs—Ringer bicarbonate containing 1 mg/ml glucose, adjusted to pH 7.4, with a

mixture of 5% CO_2—95% N_2 in the gas phase was used in the glycolysis experiments.

All of the other measurements — also in experiments to be discussed later — were carried out on skin slices, as we could by no means succeed in homogenizing skin.

Neither in the Waring-blendor nor in the Potter homogenizer using powdered quartz, could the fibre structure of skin be disintegrated properly. Only techniques yielding a surviving, more or less intact, metabolism could be considered. Finally the selected technique was the following: skin pressed between two sheets of plexiglass was cut at great speed into thin threads, then further minced with scissors. Thus, after considerable practice we succeeded in obtaining curves of good reproducibility, which could not be improved by further mincing. The time elapsing from sample excision to zero minute of readings, was 40 to 50 minutes.

The evaluation of the results shows that endogenous respiration and anaerobic glycolysis of rat skin exhibit regional differences similar to wound healing registered macroscopically. Both have a higher rate on the cranial than on the caudal part of the back. The statistical evaluation of some groups is given in Table 7. Inspection of the table reveals that cranio-caudal differences in glycolysis are consistently measured, independent of distance from the middle line, differences being significant in each case ($P < 1$, 2, respectively).

Difference of endogenous respiration medially, between nape and sacrum, is significant in all cases ($P < 1$). From the middle-line sidewards, nevertheless,

Table 8

Cranio-caudal differences of endogenous respiration and anaerobic glycolysis of rat skin

A. Endogenous respiration

Q_{O_2} (μl oxygen/mg wet tissue/hour)

	shoulder	rump	nape	sacrum
Average	0.415	0.351	0.378	0.226
Difference	0.064		0.152	
Number of experiments	10	10	10	9
P %	>20		<1	

B. Anaerobic glycolysis

$Q^{N_2}_{CO_2}$ (μl CO_2/mg wet tissue/hour)

	shoulder	rump	nape	sacrum
Average	0.604	0.407	0.608	0.414
Difference	0.197		0.194	
Number of experiments	11	11	11	10
P %	<1		<2	

Table 9

Comparison of endogenous respiration and anaerobic glycolysis of the skin of young and adult rats

A. Young rats

	Q_{O_2}		$Q^{N_2}_{CO_2}$		Weight of animals g
	nape	sacrum	nape	sacrum	
1.	0.29	0.20	0.39	0.35	60
2.	0.37	0.34	—	—	65
3.	0.39	—	0.42	0.33	55
4.	0.40	0.37	0.51	0.35	58
5.	0.42	0.30	0.29	0.21	60
Average	0.37	0.30	0.40	0.31	

B. Adult rats

	Q_{O_2}		$Q^{N_2}_{CO_2}$		Weight of animals g
	nape	sacrum	nape	sacrum	
1.	0.31	0.22	0.43	0.31	145
2.	0.34	0.32	0.39	0.28	200
3.	0.30	0.25	0.34	0.18	220
4.	0.22	0.18	0.25	0.14	260
5.	0.28	0.16	0.24	0.17	200
Average	0.29	0.23	0.37	0.22	

Q values calculated on wet weight basis.

between the shoulder and luteal region, the difference is statistically not significant (Table 8*A*). Measurements of endogenous respiration and anaerobic glycolysis were also carried out on the skin of young rats, in which respiration is generally higher than in adult animals according to the literature [18].

As it was to be presumed, Q values were higher in animals, weighing from 50 to 60 grams, than in adults, nevertheless cranio-caudal differences were also distinct. Q_{O_2} and $Q^{N_2}_{CO_2}$ values of five young and adult animals are denoted in Table 9*A*—*B*. Experiments were carried out simultaneously, so as to avoid possible seasonal variations interfering with the comparison.

Experiments comparing aerobic glycolysis of different areas along a longitudinal somatic axis were also undertaken. These experiments failed to give an answer to the question raised, because aerobic glycolysis of rat skin is of such negligible intensity that regional differences were not measurable with adequate reliability. Intensity of aerobic glycolysis in rat skin is 1—1.5 microgram lactic acid production (hour) mg skin, the corresponding glucose utilization is not stoichiometric: 2 microgram (hour) mg. Aerobic glycolysis measured lactic acid determination according to Barker and Summerson [2] and simultaneous glucose estimation by the method of Nelson [15]. The

experiments were but of methodological interest. Slices were incubated at 37 °C in the presence of pure oxygen. The method used for stopping the reaction at desired intervals permits the kinetic study of processes other than glycolysis. Incubation was undertaken in homogenizer tubes according to Potter, the reaction was stopped by homogenizing with cold perchloric acid.

Preliminary experiments were carried out concerning the incorporation of radioactive P^{32}. Adult rats were given 100 Curie P^{32} intraperitoneally, skin was extracted and fractionated according to Schmidt and Tannhäuser.

Incorporation into inorganic phosphorus shows a higher rate cranially, corresponding to the blood supply. The nucleic acid content is also considerably higher cranially, suggesting a greater cell density, which is supported by histological findings. Moreover, specific activity of nucleic acids appeared to be higher cranially: 6.80—4.35 imp/mg NA phosphorus and 4.17—2.40 imp/mg NA phosphorus, respectively, on the caudal part. These incorporation experiments being still in a preliminary stage, we intend to continue the work. After reporting on our metabolic studies on intact skin we will now summarize our studies made during the healing of wounds.

We attempted to gain material for the study of local metabolism by various methods. The excised regenerate was first subjected to histological examination, to check how far we had achieved uniform sampling *i.e.* with what reproducibility the sampling method worked. Numerous methods had to be rejected after these preliminary investigations. Thus, we attempted to remove tissues from the healing wound by curettage. We attempted to excise it according to Paul and co-workers [18]. We tested the method according to Schilling and co-workers [25] using sutured wire mesh. Histological examination always showed the removed tissues to contain varying amounts of granulation tissue, detritus, exudate, etc. Hence these methods had to be discarded. We did not even try the polyvinyl sponge implant test method of Edwards and co-workers [9] as we assumed that this amount of foreign body would interfere with the progress of healing, and so doubted the reliability of the results. We also tried to apply the wound margin eversion method published in Hungary by Lamm and Rudas [12] but we did not succeed in obtaining a sufficient amount of tissue for investigation, as was published by the author.

After several attempts we decided to resort to the study of energy producing metabolic processes in the intact skin adjacent to the wound, during a few days following the injury. We were compelled to do so by the technical difficulties described above and, on the other hand, we thought that healing, *i.e.* restitutive synthesis starts from the intact tissue around the wound. Previously histological studies have already been carried out on the wound margin, based on similar reasoning [1, 22].

The wounds were made inflicted under Nembutal anesthesia, respiration and glycolysis of the skin sample excised on wounding was considered as normal value. At certain intervals after wounding the respiration and anaerobic glycolysis of a 1 mm wide skin strip immediately surrounding the wound was determined in slices, excising the skin strip on the wound edge also under Nembutal anesthesia.

Numerous data are known about the biphasic nature of wound healing [14]. It is a generally accepted opinion that the first stage of wound healing is a regressive, metabolic period, the so-called R phase, which is followed by a progressive P phase of enhanced activity, differentiation and synthesis.

In recent years considerable experimental work has been undertaken concerning the various changes occurring during the R—P phases of wound healing, using not only histological but biochemical methods as well. Changes of respiratory and glycolytic activity have also been described by several authors. In 1947 Paul and co-workers [18] reported that endogenous respiration of rat wounds increases to the fivefold of the normal value on the sixth day, subsequently decreasing. A result gained not on skin is that of O'Brien [17] who in 1959 found on the regenerate of the earth worm that glycolysis reaches a minimum, respiration a maximum on the tenth day. It was published by Rossiter and Glark [24] in 1944 that simultaneously with the decrease of

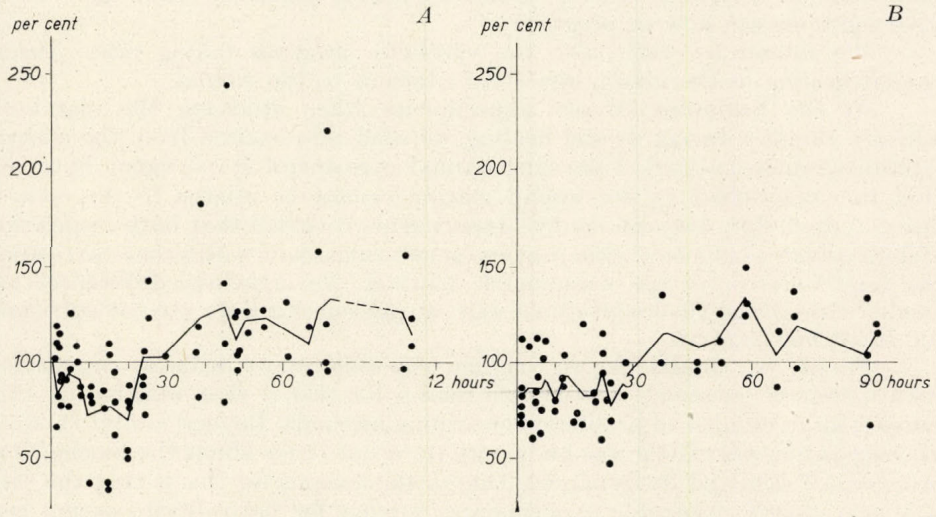


Fig. 20. Wound healing in albino rat. *A* — Endogenous respiration and *B* — anaerobic glycolysis of the intact 1 mm wide strip adjacent to the wound. Q values are represented in the percentage of the values of freshly excised intact skin. Wet weight basis. *A* — Slices incubated in Krebs—Ringer phosphate adjusted to pH 7.4. Gas phase 100% oxygen. *B* — Slices incubated in Krebs—Ringer bicarbonate containing 1 mg/ml glucose, adjusted to pH 7.4, mixture of 5% CO_2 + 95% N_2 in the gas phase

respiration glycolysis increases. According to Needham [16] local metabolism is rather more glycolytic in the R phase and more oxidative in the P phase.

During our studies we also registered activity changes occurring regularly in the immediate surrounding of the wound, but we found the course of the phenomenon in this tissue to differ from that described in the literature for the regenerate itself. In the intact skin strip around the wound, endogenous respiration and anaerobic glycolysis both show a 24 hours decrease, followed by a subsequent increase above the normal level, and remain at that level during the four, respectively, five days it was studied. This phenomenon is seen around the wounds of both nape and sacral regions. In Fig. 20 data are graphed in relationship to the intact skin of the same site, excised on wounding.

Despite the variations encountered in these experiments, it can clearly be seen, that within the first day values cumulate to beneath 100% *i.e.* normal values of intact skin, whereas after 24 hours they exceed this value. The drawn

line denotes average values. To the evaluation of the graph it must be added, that of the strips studied arbitrarily in 1 mm width, we do not know in what ratio they contain intact cells not participating in the wound healing processes. Their normal activity of respiration and anaerobic glycolysis obviously masks and shifts the points to an unknown measure, towards normal values. If we had better means to separate the normal skin components from those participating in the reparative process, we would get a more pronounced deviation from the norm, and a steeper transition with time, as the metabolism of normal cells present also in a way as to obscure the transition around 24 hours.

As yet we know nothing about the causes and mechanism of this transient inhibition of energy producing processes taking place the first day. These investigations are now in progress.

We intend to study also the synthetic progress taking place during wound healing in the intact skin strip adjacent to the wound.

At the beginning of our experiments, when studying the metabolic activity changes during wound healing, we used skin excised from the contralateral, symmetrical part of the same animal as a control. It was found, however, that the metabolism of the wound margin cannot be related to the contralateral intact skin, because control experiments revealed that both respiration and glycolysis of identical skin regions on two sides show a difference exceeding the limit of error of the manometric method. Nevertheless, differences are smaller than along the longitudinal axis, at present our data are not sufficient for statistical analysis.

Finally we should like to explain why this report deals so extensively with technical questions. The simple reason for this is that working on skin raises a large number of problems concerning methods. Beyond analytical data of composition very little was known up to recent times about the biochemical processes of skin and its dynamics. One of the reasons for this is that the skin is a very poorly accessible experimental subject for both disintegration and precipitation. Without elaboration of adequate methods, a detailed study of the processes involved was practically impossible. This structure-induced difficulty justifies the great attention paid to methodologic problems.

Discussion

The results of our investigations permit to draw the following conclusions:

It was demonstrated on wounds inflicted along two cranio-caudal axes on the dorsal side (middle line and shoulder-rump) of three kinds of mammals (guinea pig, albino rat, dog) that the cranial woundings heal in a much shorter time than the caudal ones.

Of all the somatic parts tested, abdominal wounds heal the most rapidly.

It was established that wound healing is influenced by age, in that wounds of young animals heal generally more rapidly, but the cranio-caudal axial gradient exists at all ages, the cranio-caudal difference being greater in young animals than in adults.

It was demonstrated that wound healing rate is not measurably influenced by multiple wounding of the same animal.

It was established that the axial gradient found on the dorsal part of the body cannot be demonstrated on the ventral side.

It was established that sex and skin pigmentation of the animals, or the seasons have no perceivable effect on wound healing.

It was demonstrated that there is a difference in the healing of wounds made on symmetrical (right and left side) skin areas in the same animal.

The course of wound healing in fish could only be followed up by histological methods.

In vitro metabolic studies showed that the skin regions exhibiting faster healing have a higher anaerobic glycolysis and also, in the middle line of the back, a higher rate of respiration. Abdominal skin seems to have the highest metabolism. Exploratory isotopic experiments seem to indicate that both incorporation rate of labelled phosphorus and turnover rate of nucleic acids are higher in the more cranial skin areas.

Histological studies showed the epithel to be broader, more cellular and far more papillated in the skin areas healing better and possessing a higher rate of metabolism than in the regions healing more slowly. On histological slices it could be demonstrated that epithelization of more cranial wounds takes place more quickly than of those situated more caudally, also in fish.

Thus, a certain parallelism could be observed between wound healing rate, metabolic activity and histological structure.

Skin temperature in various regions also showed a tendency to craniocaudal decrease.

There was a striking interconnection between function and structure.

The question arises, what is the role of circulation in the observed phenomena. According to our clinical experiences (good circulation of the head, poor blood supply of the lower limbs) the importance of circulation is unquestionable. The extensive papillarity of cranial skin regions demonstrated in the histological slides also points to this, as the papillar layer is the site of the circulatory system. It is very likely that this plays a part in the observed differences in phosphorus incorporation. It is beyond doubt that a high metabolic rate requires a rich circulation, and vice versa a better circulation permits a more intensive metabolism.

The question to be answered is, what do we consider to be primary. We probably have to do here with a phylogenetic functional accomodation.

Every living being developed constant correlation and interaction with the outside world. Its essential interest was to constantly obtain information from its environment, to perceive immediately every change in the surroundings and to react in the shortest possible time to impulses received. Thus, it is conceivable that for mobile living beings, the animals, particularly for those with a definitely orientated motion, the instruments for obtaining informations from the outside world, *i.e.* the sense organs were developed mostly on those parts of the body which were nearest to the direction of movement, at the cranial pole. The most cranial segmental nerve centres, hitherto more or less uniform in importance and size with the others, gradually acquired a leading role. This differentiated and multiform function of the cranial pole requires a high metabolic rate, a correspondingly more intensive blood supply. A certain role might be ascribed to the fact that obviously the part of body lying in the direction of motion might have been injured the more frequently.

According to this reasoning, the higher metabolic rate, more rapid wound healing and better circulation of cranial skin regions must be considered as a functional accomodation developed in the course of phylogenesis.

Our experimental results also indicate that in this correlation an exclusive and primary role cannot be ascribed to circulation. We studied metabolic rates in excised, surviving skin samples, already excluded from circulation, and also here an axial gradient was to be found.

On this basis, we might attempt to answer the question raised in the introduction.

We believe it can safely be stated that it seems probable, that the good wound healing on the head, the poor wound healing on the legs observed in medical practice day by day on human beings, cannot be ascribed solely to circulatory conditions due to man's vertical posture and from the resulting impeded venous circulation of the lower extremities. These factors undoubtedly play a role but we believe them to be secondary. In our opinion the fundamental causes are biological factors more ancient than man. Certain axial gradients seem to be discernible, not only on invertebratae but on higher animals: vertebratae, mammals, and also on humans. Of course, it remains to be seen to what extent this is analogous to the gradients observed in lower animals.

We think an attempt should be made to find an explanation of the phenomenon that axial gradients found in wound healing, metabolic rate and skin temperature can be observed only on the dorsal part of the body of humans, as well as on those of mammals used for the experiments. This phenomenon might be explained by embryonal development.

The dorsal part of the body shows marked segmental organisation during ontogenesis (dermatomes, myotomes). This is manifested on all vertebrates even on fully developed individuals, concerning certain arteries and nerves.

It would seem that a decrease of potential in cranio-caudal direction is a characteristic feature of segmental organisms built according to a symmetry axis and is present on those parts of the human body which show segmental development during ontogenesis as a phylogenetic heritage.

We think the results of our experiments permit us to state that the healing rate of wounds, naturally ceteris paribus *i.e.* under normal conditions when wound healing is not influenced by any external factors or internal pathological processes, is primarily dependent on the biological qualities of the injured tissue, also manifested in the metabolic rate of the latter. There is a parallelism between metabolic activity, temperature and wound healing rate.

We wish to add a few words referring to the lateral differences found during our studies. These experiments are still in progress, so we cannot talk of conclusive results, only assumptions can be outlined. The question arose whether the differences observed between the two sides of the body are in relation to lateral dominance. Hitherto in our experiments no prevalence in favour of one side or the other could be demonstrated. It occurred with about equal frequency that wound healing rate and metabolic rate were higher on the left or right side.

In the literature we find that unilateral dominance might exist in mammals. It seems to be rather an exception for both sides to be equivalent. Data suggest that the distribution of dominance between the two sides is equal [7, 10, 30].

In future experiments we intend to clarify whether the more rapid wound healing and higher metabolic rate is in correlation with unilateral dominance.

According to common knowledge among humans, right-handed individuals outnumber by far the minority of left-handed ones. We want to forestall the question of how to explain this if we accept not based on our own experiments but at present only on literary data, that among mammals the dominancy of one or the other side occurs with equal frequency.

In this respect we have to state that it depends to a great extent on the methods of examination, to what percentage humans are found to be right- or left-handed, *i.e.* in what percentage is the left, resp. right hemisphere dominant. If we take into consideration the so-called latent sinistrality too, then we arrive at data as to frequency of sinistrality varying between very wide limits [23]. There are far more left-handed individuals among small children than among adults [23]. This suggests that the prevalence of dexterity is a social phenomenon, the result of training and co-operative work.

We think the first part of the above conclusions, based on our direct experimental results, seems to be conclusively demonstrated. The latter are of hypothetical nature. These are to be considered as a working hypothesis which will either be corroborated or rejected in the course of our further studies.

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Contributory Discussion

Prof. Tokin: Dr. Szántó distinguished between young and old rats. I should like him to tell what he means by young and old in this connection.

Dr. Szántó: Young rats weighed 60 to 80 g; adult, sexually mature but not old animals had a body weight between 120 and 150 g, while rats with a body weight of 300 to 350 g and guinea pigs with one of 800 g were regarded as old.

Prof. Tokin: I experienced the same problem. We found that although classification according to age did not affect the gradient, the numbers of the days of absolute healing overlapped so that cranial recovery in old animals took the same time as caudal recovery in young ones. While metabolic processes were different in animals of different age classes, nobody spoke of age in connection with differences concerning gradient. I hold, therefore, that distinction according to age is very important; body weight may suffice in the case of young animals, but should not be taken as a measure in old ones. I should, therefore, like to receive information concerning the principle of age selection in those cases where age has not been mentioned.

Dr. Szántó: We selected our animals according to weight and not according to age. In the series of experiments in which the metabolism of the skin of "young" animals (50 to 60 g) and "old" rats (200 g) was compared, the weight of animals used for all other metabolic tests amounted to 120—200 g.

Dr. Sugár: What was the age in hours or days of the granulation tissue in which increase in glycolysis and respiration was compared?

Dr. Szántó: No comparisons were made in granulation tissue because histological examination, made in the course of preliminary experiments, had shown that we were unable to remove reproducible granulation tissue. Measurements were effected in strips of intact skin around the wound, within a few days after the wound had been inflicted.

Dr. KELLNER: I do not see clearly: both the strip and the wound were involved or the strip alone?

Dr. SZÁNTÓ: The whole area around the wound was excised with a band of skin that measured about 1 cm in width, and the wound itself was dropped; after having cleaned the inner surface by means of excoriation, we excised a band of about 1 mm around the wound. It was for this reason that we concerned ourselves with the initial period only, since later it was impossible to remove a sharply circumscribed strip of 1 mm from around the wound.

Dr. KELLNER: The principle of manipulating the marginal parts of the wound seems to me quite correct; that the centre drops out within 5 days is natural, since there is a defect at that point covered with crust. However, I find the time too short, and should like to know why the experiment did not last longer. Any technical difficulty in this respect ought to have been overcome by some means or other, for — I venture to say — the experiments would have become interesting just after this point.

Dr. TSANEV: I am much interested in the paper of Dr. Szántó and his associates. Having conducted experiments with the same animals, I agree with him that wounds heal slower in the sacral than in the cervical area. I cannot, however, subscribe to the contention that one is dealing here with a problem of metabolic gradient as understood by Child. Decrease in the size of wound is not only due to cellular proliferation as is the growth of a blastema or the process of regeneration in some part of the body. Our explanation of the discrepancy between sacrum and neck in respect of wound-healing is different. The formation of granulation tissue depends largely on the underlying adipose tissue. Whereas there is much adipose tissue on the neck where the skin is but loosely attached to the underlying muscles, there is less fat and less loose connective tissue in the sacral area, so that the growth of granulation tissue and a concentric diminution of the wound occur at a quicker rate in the cervical area. Another point at issue is the state of hair follicles. The growth of hair represents a cycle of changes, and I should like to know whether it was taken into account in the experiments. The very metabolic differences may depend on the stage of hair-follicle growth. It is stated in the paper that the number of cells was higher in the skin of the neck than in the skin of equal dry weight obtained from the sacral area. I would suggest to adopt rather the amount of DNA as a standard of reference, since there are more cells in the larger than in the less developed hair follicles.

Dr. CSABA: I should like to know the comparative importance of the axial gradient and the phylogenetically developed functional adaptation. This problem is actually raised in the paper itself. That functional adaptation may play a certain role is supported by the fact that — to our knowledge — wounds on the hand of humans heal rather quickly, a phenomenon which would prove that this area, one much exposed to traumatization, has a tendency to adaptation by having developed a strong power of regeneration. It is known that the skin is much thicker on the palm of the hand than — say — on the back. It appears from the paper that skin obtained from the cervical area of rats is thicker than that taken from the sacrum: this would point to that the area in question, having become functionally adapted, possesses a comparatively higher capacity of regeneration. It might be worth while to ascertain differences of regenerative power due to right- and left-handedness. Human subjects are mostly right-handed which must be due to a phylogenetically developed hereditary trait. Far be it from me to doubt the existence of axial gradient in mammals and human beings; all I should like to find out is this: somatic embryogenesis diminishes as we climb up the phylogenetic ladder, while the regenerative capacity remains unchanged or even increases; is it, then, possible that the significance of the axial gradient diminishes in mammals whereas the phylogenetically developed power of adaptation gains in momentum to become dominant?

Prof. MÖDLINGER: Relying on the evidence of personal observations, I want to make a few comments on the axial gradient of rats. We produced, for experimental purposes, hybrids by crossing white with black rats, and these hybrids were highly interesting in respect of pilar pigmentation. Some were white, some black and others dappled. The latter displayed strong pigmentation on the head, around the neck and the shoulder, further along the backbone, while the hair was white in other parts of the body. The pigmented hair along the spinal column was suggestive of the pigmented band of wild horses, called "Altstrich" by German authors. Pigmentation of this kind is restricted to the area above the spinal column, and it might be worth while to institute metabolic investigations, further experiments of regeneration and wound healing in this part of hybrid rats.

As regards now axial gradient in fishes, it is commonly known that, in certain species, the epidermis measures 65 μ thick on the head and only 43 μ in the caudal region

of the back. Conditions of wound healing were highly interesting but it would first be necessary to analyse the structure of fish epidermis. A thin cuticular layer constitutes the outer envelope beneath which the epidermal cells are situated. The arrangement of these cells is different from that usual in other vertebrates, as there are no tonofibrils but — on the contrary — intercellular cavities can be observed under the microscope, a phenomenon indicative of high cellular mobility. These cavities between the cells would be filled with the substance secreted by one of the three kinds of epithelial glands present in the skin of fishes. Epithelial proliferation, as seen on the 4th day, was quite impressive. The males (and, in certain species, also the females) affect the intensity of wound healing. There exist, in respect of urodeles, experimental data to show that a higher number of wounds accelerates the rate of healing. My third question touches upon the title of Dr. Szántó's paper which might give rise to objections. It is, of course, acceptable if gradient is understood only to mean difference. However, gradient in the sense of Child's classic experiments means something different. According to him, the character of the gradient changes in proportion to the structure of the organisms becoming more intricate, and he used the term gradient only in connection with organisms of comparatively low structures. He and his co-workers applied this term in connection with the higher vertebrates to embryos only.

Prof. KELLNER: A considerable part of Dr. Szántó's lecture consisted in the discussion of methodical questions. I should like to expatiate upon a few problems of this nature. The age of the test animals is of Cyprinidae and Salmonidae develop so-called pearl organs during the period of spawning, and the structure of these organs is very similar to the said epithelial proliferation. These organs develop under hormonal influence so that a certain correlation may exist between hormonal regulation and proliferation. It should be noted that pearl organs are repeatedly formed during one and the same spawning period in certain cyprinoid fishes, *e.g.* in the goldfish; the horny layer becomes detached, and the organs are formed anew, so that the substance in question would deserve further investigation from the angle of wound healing.

Prof. TOKIN: I want to ask Dr. Szántó three questions. The first is that the adult organism does not indicate the biological value of the old organism. It would be advisable to supplement the highly valuable studies by the use of really old rats, and the extirpation of the sexual apparatus should also be considered in connection with the experiments. My second question is whether the number of wounds has any fundamental importance, since — according to a basic idea of the lecture — the gradient, *i.e.* the difference between the cervical and sacral region in respect of subcrustal wound healing, diminishes with advancing age. Investigations should be continued along this line. Large wounds covered by crust heal by means of another mechanism than linear wounds similarly covered. I would suggest that, besides wounds made by means of trephine, also wounds made by simple incision be investigated. I want to emphasize that such investigations would have to be accompanied by simultaneous histological analyses. I do not wish to discuss the question whether one is dealing here with a phenomenon equivalent to the gradient as interpreted by Child. For all my criticism of the applied method there can be no doubt that mammals display a fundamental difference between the head and the sacral area as regards the healing of wounds, a difference of decisive importance.

Dr. TÖRÖK: The paper under discussion contained a reference to the possibility that the observed behaviour of the dorsum (where gradient has an undoubtedly axial direction) may be correlated with dorsal differentiation manifesting itself as early as the embryonic stage. I should like to restrict this phenomenon to the nervous system, since its differentiation constitutes the most notable morphogenic phenomenon occurring on the dorsal side in the course of embryonic development.

My observations agree with those of other workers according to whom mitosis and processes of growth are stimulated by the neurohumoral activity of the nervous system. Accumulation of nerve cells in my test animals was invariably followed by intensive growth at the same point. The substance released by the nerve cells produces its growth-promoting effect by spreading from cell to cell or by diffusion into the interstitial fluid. Nerve cells may have a similar function in vertebrates.

Dr. SUGÁR: I want to repeat what has already been mentioned in the course of the discussion, *i.e.* that the cyclic exchange of hair follicles is a problem that must not be disregarded.

Dr. SZÁNTÓ: As regards Dr. Tsanev's remark that the healing of wounds is not connected with metabolic gradients as understood by Child, I suggest to reply later. He thinks that differences in the subcutaneous connective tissue are of importance. While it is undeniable that such differences do exist in mammals, for instance between

the occiput and the sacral area, they are considerably less conspicuous between the shoulder and the sacral area. It must be remembered that we demonstrated the metabolic differences on surviving fragments of excised skin deprived of connective tissue, so that differences in the subcutaneous connective tissue cannot have played a fundamental and decisive role. I am, at the present stage of our experiments, not in a position to offer accurate information regarding the cyclic exchange of hair follicles. I am grateful for the comments made in this respect, and we shall not fail to investigate the matter. This will, at the same time, settle the same problem raised by Dr. Sugár. I have also to thank Dr. Tsanev for his useful suggestion to use DNA as a basis of reference. We are fully aware of the usefulness of the procedure and have decided to apply it in future experiments.

As to Dr. Csaba's question concerning the comparative importance of the axial gradient and phylogenic evolution, I should like to know — I beg to be excused for replying to a question by another question — whether the axial gradient appeared independently of phylogenic factors or whether it was just the outcome of evolution.

Dr. CSABA: The axial gradient is obviously a result of evolutionary development, but it is a very early pattern. It may, therefore, be on the decline in mammals. Functional changes are characteristic features of phylogeny.

Dr. SZÁNTÓ: According to my — biologically surely inexpert — opinion, axial gradient represents phylogenetically evolved functional adaptation like any other such phenomenon. I fail to perceive any sharp difference in this respect. The core of the question under discussion is whether the two things intersect or whether they are identical. I think we are speaking of the same phenomenon, and that all misunderstanding derives from the fact that it is not customary to use this term in connection with higher animals. Healing of wounds on the hand has also been mentioned. Surgical observation shows it to be undoubtedly more rapid than on the lower extremities. Wounds of the head heal at a still more rapid rate than those of the hands. The thickness of the skin cannot serve as a criterion in this respect: skin is thick not merely on the palm, that of the sole is still thicker and plantar wounds heal slowly, nevertheless. I do not think that the thickness of the skin, in itself, is the decisive factor. The question of left- and right-handedness has likewise been raised: I contented myself with just touching upon it in the lecture since our knowledge in this respect is still fragmentary. All I want to observe regarding this matter is that literary data show the predominance of the right hand to be socially rather than biologically determined. The more examinations are conducted at an early age the more left-handedness is revealed, especially if examinations extend to latent left-handedness as well. It would be worth while looking into the matter more thoroughly: it would seem that, genetically, man — like other mammals — is not more right-handed than left-handed.

I am indebted to Prof. Mödlinger for his valuable suggestions.

As regards now Prof. Tokin's comments I want to emphasize that a determination of the rate of wound healing in young and old animals was not the primary object of our investigations. Our fundamental working hypothesis was that the cranio-caudal difference in respect of wound healing represented a universal biological phenomenon irrespective of age and sex. As the problem of the predominance of this or that side emerged as an interesting side issue in the course of our study, so did the matter of a lessening of the axial gradient with advancing age crop up during the investigations. We suggest to examine the matter in experiments on really old animals. Regarding the question as to whether the number of wounds affects the rate of healing I cannot but repeat that multiple wounding did not seem to affect healing in our experiments. My following remarks regarding Child's gradient are meant as a reply to Dr. Tsanev's question also. Not feeling myself to be sufficiently trained in biology I refrained from committing myself on the question of whether our investigations justify us in extending Child's theory of gradients to mammals. However, observed facts seem to confirm the view that gradients along certain axes do exist in higher animals in respect of wound healing, local metabolism (meaning dermal temperature and anaerobic glycolysis) and histological structure.

Prof. Kellner raised a few problems of methodics. There is no doubt that parallel histological, especially histochemical, analyses are necessary.

As regards now Dr. Török's remarks concerning the prominent role of the dorsal side, I must agree with the statement that the location of the central nervous system is of signal importance. While the back shows a segmental embryonic development both in man and mammals, the abdominal side develops by means of issuing processes from above and from below, *i.e.* non-segmentally. This would, in my opinion, explain why the phenomena under review appear on the dorsal aspect of the body only.

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EXPERIMENTAL ASEPTIC NECROSIS OF THE BONE

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Synopsis

The authors describe their new method for creating experimental aseptic bone necrosis. The blood vessels and nerves having been severed, one of the carpal bones of the dog was replaced into the socket in its original anatomical state. Between two weeks and sixteen months following operation the formation of aseptic bone necrosis and the duration of regenation were studied in thirty-two cases. Beside bone necrosis cartilaginous tissue was only slightly damaged in an early stage. As a consequence of necrosis the spongy bone crushed and deformed. The revascularization starting from the direction of the ligaments inserted on the bone played an important part during regeneration. Meanwhile a repeated transformation of the bony substance was observed which can be explained by the various functional uses. With this new method radiologic and pathologic changes similar to human aseptic bone necrosis can be called forth, enabling us to study the pathology and clinical treatment of aseptic bone necrosis.

Introduction

The concept of aseptic necrosis of the bone originated from Axhausen [1] who was the first to make detailed studies of the problem and showed that König's osteochondritis dissecans, also Kienböck, Köhler and Perthes diseases having the same pathohistological patterns. In each of these conditions necrosi] of the osseous tissue is the dominating feature, beside which the inflammatory phenomena are slight or completely absent. According to Axhausen the necrosis of the bone may be traced back to an impairment of circulation, which in turn is suggested to be due to an embolic occlusion of the blood vessels. According to Rieger [11] vascular obstruction is due to fatty embolism. Other authors claim that the vascular occlusion is caused by an inflammation of the vascular wall, which shows the picture of endangiitis obliterans [5, 6]. According to still other views the circulatory disturbance resulting in necrosis of the bone tissue arises from nervous effects following repeated traumatization [8]. A role has also been ascribed to overstrain of the bone [4, 12, 15]. Rutishauser [13] has tried to prove the role of overstrain in animal experiments. More recently, human and experimental evidence has been accumulating to show that aseptic necrosis of the bone is traumatic in origin, with a particularly important role played by repeated microtraumata. In the experiments trying to bring about changes similar to aseptic necrosis of the bone as it occurs in man, traumatization is most commonly employed for this purpose [2, 9, 14].

In our own experimental investigations we have induced aseptic necrosis of the carpal bones. The model we have developed bears resemblance to the

sterile (aseptic) necrosis of the os lunatum malacia of Kienböck or to those following fractures of the scaphoid bone. When evolving the experimental model, we intended to create ways and means by which we could study aseptic bone necrosis and the processes of regeneration taking place by means of histological methods, and thus, to obtain data as to the dynamics of the process of regeneration. The other aim was to create an experimental model that closely resembles aseptic necrosis in all respects as it occurs in man, and that may be used for elaborating and appraising the effective methods of treatment.

Experiments

The experimental object in which we induced aseptic necrosis was one of the carpal bones of the dog; the central radiocarpal bone. This bone is covered by cartilage on its proximal, distal and ulnar surface. On the dorsal surface there is a thin area covered by periosteum for the insertion of the dorsal capsular ligament. On the volar-radial surface there is a minute tuberosity for the insertion of the tendon of one of the volar, flexor muscles. On operating, the bone was exposed from the dorso-radial longitudinal approach, the capsular ligament and the tendon were cut, and the bone was taken out from its surroundings, depriving it thereby from all its vascular and nervous connections. The bone was then immediately replaced to its original anatomical position, the capsular ligament was closed by suture, the tendon insertion was not reconstructed. The surgical wound was closed per primam and a splint coated on the inside with paper wadding was placed on the limb. Two or three weeks later the animals operated on were using their operated limbs normally.

The operation outlined above was performed on both forefeet of 16 dogs, thus, we had a total of 32 bones which had been replaced into their original anatomical position after severing vascular and nervous connections. The single bones were taken out for study after different intervals of time: 2 weeks, 1 month, 2 months, 4 months, 6 months and 9 months after operation, for examining 4—4 carpal bones at every point of study. In 8 cases the operated bones were removed and studied as late as 14 to 16 months after operation.

The removed bones were fixed in 8 per cent formaldehyde solution, decalcinated using an electric method, cut up into 4 to 8 parts by making parallel sections and were studied histologically after embedding them in paraffine.

The most conspicuous histopathological change found in the bones removed two weeks postoperatively was the excessive, often complete necrosis. In the area of the necrosed bone-trabeculae, the bone cells in the cavities of the osteocytes showed no staining, but sometimes the contours of the osteocyte could be detected. Necrosis was not only restricted to the bone, but it also involved the cellular elements of the bone marrow. More often only the outlines of the fibrous substance were visible, the cells had perished completely (Fig. 1). In some cases necrosis was not complete, but islets of varying size of intact, well-staining bone tissue could be seen. In such cases it is assumed that after the bone had been put back into its original place, revascularization must have been so favourable that single islets could remain free from necrosis. It was remarkable that, just as it had been observed in man too, the cartilage coat of the extensively necrosed bone always remained intact. The necrotic subchondral bone and the living cartilage were clearly distinct from each other

(Fig. 2). The shape and staining of the cartilage cells were normal everywhere. Thus, the cartilage does not perish together with the bone, but the necrotic bone substance is always covered by living, intact cartilage. This means that the cartilage, which is a bradytrophic tissue requiring little nutrition [3], has retained its vitality in the articular cavity even after such a drastic intervention and such a serious interference with physiological relations.

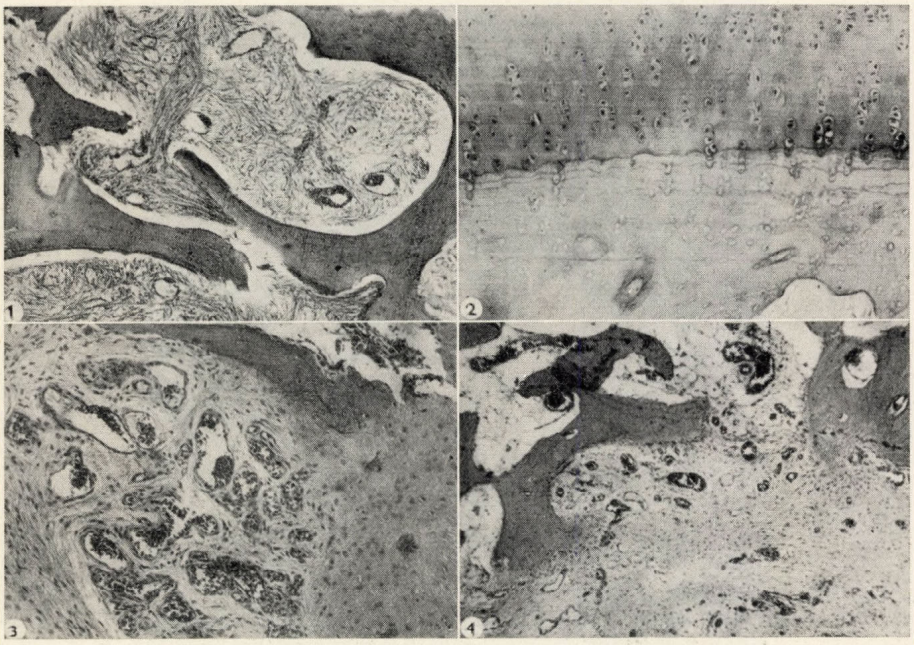

Fig. 1. Completely necrosed and disintegrated bony trabeculae, with fibrous, necrobiotic connective tissue in between, in which mainly the fibrous substance is conspicuous, cells are absent. *Fig. 2.* Total necrosis of subchondral bone, having an intact cartilage. The living and necrotic tissues are sharply delineated from one another. *Fig. 3.* Proliferation of densely packed capillaries in the area adjacent to the regenerating corticalis. *Fig. 4.* Early phase of regeneration. Richly capillarized granulation tissue, penetrating through the interspaces of the corticalis

The necrosed bone had become fragile and often lost its shape as a result of the forces acting on it; it was often crushed by the pressure exerted on it by adjacent bones. For this reason microscopic bone debris could be found at many sites. Such microscopic sequestra remained observable for a long time in the newly formed bone tissue during subsequent regeneration. In the later stages of the experiment, when the phenomena of regeneration were much more clearly visible, on the site of the newly formed bone, the bone tissue regained its strength and in such cases functional loading changed from time to time, as a result of which the neogenesis of bone affected in a different degree. Such changes of exposure to stress and strain are also suggestive from the point of view of the repair of aseptic necrosis in man, thus, the experimental model is also similar in this respect to the human disease.

Where excessive deformities developed in the bone as a result of extensive necrosis and crushing, the cartilage layer too, was fractured. In many cases the cartilage penetrated deep into the crushed bone. Even in such cases, however, the cartilage only perished directly in the area of the fracture ends, and it began to regenerate sooner than the bone.

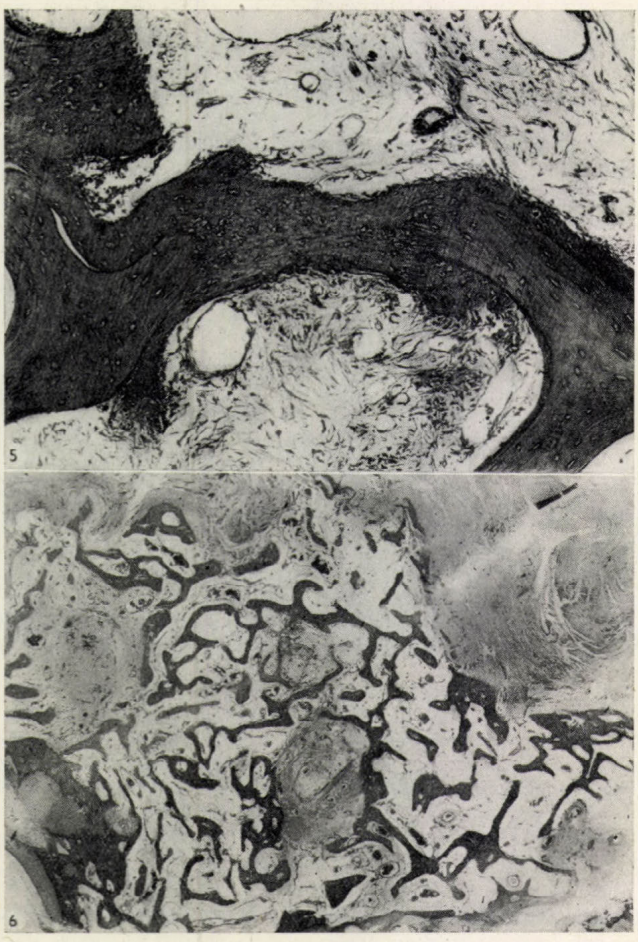

Fig. 5. The necrosed spongious trabecules are connected by young, highly cellular osteoid callus. *Fig. 6.* In the inside of the regenerating bone many foci are visible, in which regeneration proceeds slowly, because of the lack of functional stress

In the specimens examined 4 weeks after operation it could be clearly shown that with the beginning of regeneration, revascularization from the ligaments attached to the bone plays the decisive role. In these areas the activity of the mesenchymal cells can also be appraised from the increase in the number of young connective tissue cells. The amply proliferating blood vessels are similar

in several areas to those seen in the picture for angiomatosis (Figs 3 and 4). This highly vascularized tissue penetrates into the bone from several directions at one and the same time. Here intense osteoblastic apposition is visible on the surface of the young bone trabeculae, the bone marrow is composed of richly vascularized young connective tissue, and in several areas we also find newly developing patches of haematopoietic tissue. Gradually the whole necrosed area is replaced by young bone tissue. The necrosed trabeculae are removed by simple resorption, sometimes we also find evidence of osteoclastic lacunar resorption. As a result of exposure to mechanical forces and function, the newly formed trabeculae are more closely packed in some areas and less densely in others. Thereby, cancellous structure of variable density arises. The ends of the old bone trabeculae fragmented by necrosis and crushing are often united by young osteoid tissue, so that we often find structures we might call *microscopic callus* (Fig. 5).

Many months after operation, in the advanced phase of regeneration we still find foci of varying size in which the old, necrosed bone tissue is still detectable. The explanation for the persistence of such necrosed areas for so long a time seems to be that in those areas functional stress must have been minimal, in lack of functional stimuli there was no need to accelerate regeneration and, therefore, the process of regeneration proceeded at a slower rate (Fig. 6).

Such as the unequivocal histological changes in the specimens, studied a few weeks after operation, are in sharp contrast to the ones found in many a case in the more advanced stages of regeneration. To the extent the regenerating bone regains its strength, to the same extent does the functional action change in the different areas. As a result of the forces acting upon it, the bone tissue is re-built in many areas, which is often accompanied by a breaking down of the bone tissue newly built in the process of regeneration. The building and re-building to meet the requirements of stress happen side by side, so that even after several months the two processes can be studied in the same histological specimens. Not infrequently osteoclasts appear at the tip of the just developed trabeculae, which are on some sites still composed of osteoid tissue, and virtually lift out the trabecula, which has become unnecessary from the point of view of stress, from its place. At the same time, not far from that area a thin, new bony chip is formed, with intense signs of osteoblastic activity on its margins.

Besides these processes taking place inside the bone a wide variety of regenerative changes can be observed on the surface of the bone, too. In many areas the articular cartilage increases in thickness, in others it is composed of just one or two strata of cartilage cells. The subchondral bone is usually thicker than it was in the intact bone, in several areas structures similar to the ones found in Paget's disease are visible. The cortical bone develops continuously in the areas in wich there are articular surfaces. Where the bone is covered by periosteum, or where ligaments are attached to it, the cortical bone develops at a slower rate. In the areas covered by periosteum we often find only minor islets of bone, which do not merge to form a continuous cortical layer (?). It is obvious that function plays just as important a role in the development of the cortical bone as it plays in the regeneration of the cancellous bone. In some areas the cortical bone develops out of a callus of cartilagineous origin, and we find a thick cortical layer of compact structure.

Discussion

In our experiments we have succeeded in working out a method by which the pathologic and clinical features of the human aseptic necrosis of bone could be studied. The radiomorphological and histological changes were identic in all respects with those found in human disease. Though in earlier experiments necrosis of the bone could be induced, the models were less suitable for use in studies on the dynamics of regeneration, because by the methods employed the interaction between the regeneration of necrosed bone and the variable functional exposures could not be analysed. In the healing of aseptic necrosis of bone in man, the modifying influence of functional activity on regeneration should always be taken into account. Our experiments have revealed that the function has a decisive influence on regeneration: the ever changing pattern of building up and breaking down could be observed in the histopathological specimens as occurring side by side both in the cancellous and cortical bone. The effect of function on regeneration and transplantation in the case of bony and cartilagineous tissues has been confirmed in many experiments by Krompecher and co-workers [7, 10]. The histological changes which they found in their bone and cartilage transplantation experiments are absolutely identic with the ones we came to recognize as being due to an interaction between functional exposure and the process of regeneration. Our findings dealt with in the histological part of this paper concerning the role of function are also confirmed by the following experimental evidence. In some cases it has occurred that a bone removed by operation and put back again into its original place became dislocated volarly, and thus was not exposed to functional strains and stresses. Such bones did not become crushed, regenerated very slowly and in the histological slides the pattern of decomposition and rebuilding going on besides the process of building up was not observed.

Thus, according to the evidence obtained in our investigations, a definite correlation exists between the process of regeneration and functional exposure, which fact has also to have an attributed significance in the repair of aseptic necrosis of the bone as it occurs in man. By means of the experimental model evolved by us, for pathologic and clinical studies, a possibility is given of aseptic necrosis of bone by functional-pathological methods.

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Contributory Discussion

Prof. Krompecher: We saw a basically similar picture in cases of autotransplantation, further at the excision of articular surfaces chiefly in connection with homotransplantation, that is, where the material was different and did not consist of avascular, denervated, replanted bones. There are certain details which render the conclusions of the lecturers still more general. Together with Dr. Papp, we excised a wedge-shaped piece from the femur of an animal and transplanted it to the corresponding part of another animal; hence we did not perform replantation like the lecturers, but transplantation, and what is more, homotransplantation. Regeneration followed only if the surface of the transplant was less than 1 cm^2. We followed up the behaviour of homo- and autotransplanted condyles longer than two years and found, as did Dr. Peer and his coworkers, that the cartilage had remained perfectly intact and the trabeculae had broken down. Osteoid, subsequently calcifying material with readily stainable osteocytes was in some cases observed to develop laterally next to the lacunae of the remaining trabeculae. *Mutatis mutandis*, the situation was similar to that seen in connection with calluses. The importance of the functional factor was demonstrated by the experiment in which a fragment of bone, removed together with the capsule and implanted more deeply, being thus deprived of the possibility of functioning, was absorbed and destroyed even in cases of autologous transplantation. Autologous grafts transferred to the coccygeus muscle (where they had no adequate function) disintegrated, while homotransplants with functional freedom survived. Resorption takes place with the aid of osteoclasts. I am glad of these instructive experiments which have confirmed our findings made on different objects.

Dr. Peer: I want to express my gratitude to Prof. Krompecher who has both confirmed and complemented our data.

Symp. Biol. Hung., **3**, 141—148 (1963)

THE EFFECT OF PHENOXYACETIC ACID ON THE GROWTH AND HERBICIDE RESISTANCE OF PLANT TISSUES

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Synopsis

Experiments were made to grow potato tissue *in vitro*, in order to elucidate the genetical conditions of the cytoplasmic resistance to 2.4-D auxinherbicidal double action and to the herbicidal effect. In potato tissue the 2.4-D has specific auxonic and toxic effects. The results of the tests for the determination of free amino and keto acids have revealed that the effect of 2.4-D on these amounts varies from one compound to the other compound. The effect is based on a stimulation of desamination and it enriches the substrate of cell respiration, otherwise it has no influence on protein synthesis. To elucidate the mode of 2.4-D action experiments have been made to clarify the protein and phosphorus metabolism of herbicid-treated tissues. The growth-promoting effect definitely varies with the different potato varieties. As to the effect of auxinherbicid on phosphorus metabolism, it has been demonstrated in P^{32} experiments that the decrease of total P is manifest with each concentration and is based on outward diffusion. The auxinherbicid exerts its action, in a differentiated manner, on the different phosphorus fractions and the effect is reflected the most markedly by the increased activity of the lipid fraction. The problem whether the increased incorporation into the lipid fraction takes place from the culture medium or from other cellular fractions was so decided that the tissues were kept for 6 hours in a medium containing P^{32}, then transferred to a medium of normal P contents. The results indicate that the increased incorporation into the phospholipids takes place from the culture medium. From this evidence the conclusion has been drawn that also the 2.4-D concentration ensuring optimal growth is connected with a certain denaturation of the lipid membranes. The problem is being investigated in detail, in experiments on cell isolates.

Cultivation of plant tissues was begun by Vöchting in 1879. He undertook to find the smallest plant particle still showing the polarity of the whole plant, *i.e.* the ability to grow a root geotropically and shoot heliotropically. In Hungary Ottó Orsós-Orován placed as the focus of his plant tissue cultivation work, the plant wound hormones discovered in 1902 by Haberlandt. Orsós-Orován assumed the wound hormone to be identical with tyrosine [28]. Since his death virtually no plant tissue cultivation work was being done in Hungary for nearly two decades. Since 1951, our Institute is engaged in studies different from the earlier researches concerned with the cultivation of plant tissues. We are investigating those specific metabolic and biochemical-genetical properties, which characterize the growth of the excised tissues of individual organisms or of the equivalent tissues of different genera and species.

Cultures of plant tissues are more suitable for use in these studies as well as for examining the influence of specific substances on metabolic processes than are animal explants. Plant tissues can be grown relatively easily in solid, tynthetic media, and what is the most important, such growth is almost

physiological. For example, we deviate only very slightly from the physiological conditions when we grow non-assimilating tissues in cultures.

We wish to mention here only briefly our investigations which have revealed that the connection between wound hormone and tyrosine is much more complex than was believed by Orsós-Orován. According to our studies in the first place we are dealing with a secondary accumulation of tyrosine, resulting from a decrease in the activity of tyrosinase, for which the SH compounds are responsible [16]. In this work we have also elucidated the mode of action of the enzyme rhodanese [17, 18, 19]. For tissue growth, at least in the potato

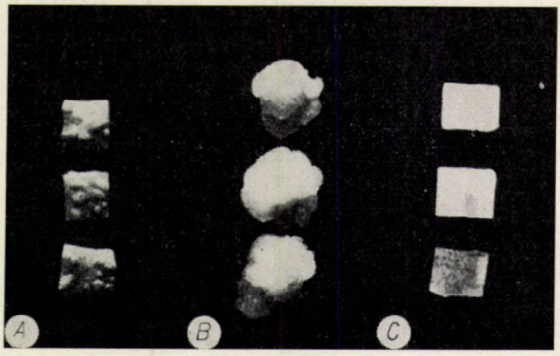

Fig. 1. Effect of different 2.4-D concentrations on the growth of potato tissue cultures. (Gül Baba potato, 16-day culture, original size). $A - 10^{-7}$ M; $B - 10^{-5}$ M; $C - 10^{-3}$ M

explant, also glutathione is responsible. Its effect can be reversed by cadmium chloride [10, 11].

We mostly used potato tissue, the cultivation of which seems to be more important from the points of view of theoretical genetics and also economics than is that of classic object (carrot, turnip, cabbage, woodbine, etc.). For decades the cultivation of potato tissue was problematic. We found that, in fact, it could not be grown in cultures with the commonly used beta-indolacetic acid in normal light [12]. Lingappa [26] arrived at the same conclusion about the same time we did. However, potato tissue could be grown in cultures in the dark, in the presence of 10^{-5} M of 2.4-dichlorophenoxyacetic acid, the well-known herbicidal agent [12]. It is still unknown why darkness is required, but it is certain that it has no bearing on the photodestruction of the growth substance (Fig. 1).

Also at the First International Plant Tissue Culture Conference held in 1954 at Briançon, most of the authors devoted the greatest attention to an expansion of cultivability to as many species as possible. This is still the trend followed by most of the major plant tissue culture research teams [22, 23, 29, 33]. We, on the other hand, have undertaken to study, under the exact condition of tissue culture, the double action of the growth regulators, of the phenoxyacetic acid derivatives, notably their growth-promoting *auxonic* and their *herbicidal* effects, and to find the genetical basis of this double mechanism.

We have determined the free amino acid and keto acid composition of the tissues and analysed how their amounts and distribution are influenced by the auxonic and herbicidal concentrations of phenoxyacetic acid. We used several thousand explants in these studies [13]. The free amino acid and keto acid studies were conducted with two-dimensional and circular chromatography, using a quantitative photometric technique and the methods of Fischer—Dörfel, Levy and Chung, as well as of Alfthan and Virtanen [20, 25, 2].

Three types of behaviour could be distinguished in the case of free amino acids:

1. Their quantity is strictly correlated with the rate of growth, but some decrease is noted at higher concentrations of 2.4-D. Here belong arginine, aspartic acid, alanine, proline, methionine and valine.

2. Their quantity changes parallelly with growth as well as with the concentration of 2.4-D. Such amino acids are glutamic acid, glutamine and the glycine-threonine-tyrosine complex.

3. Leucine and gamma-aminobutyric acid disappear completely on the fifth day of growth (Table 1).

The data for the free keto acids, determined by preparing their 2.4-dinitrophenylhydrazones [21], are in harmony with the above results. The behaviour of the alpha-keto acids is approximately the mirror image of that of the amino acids. This was particularly conspicuous in the presence of the phenoxyacetic acid concentration ensuring optimal growth, especially as far as the concentrations of pyruvic acid, oxypyruvic acid, sulfopyruvic acid and alpha-keto-glutamic acid were concerned. From this we could draw the conclusion as to the auxonic effect of phenoxyacetic acid, that the 2.4-D effect manifests itself in an *increase of desamination, i.e.* it *provides substrate for respiration* and *not for the synthesis of protein.* Since then, our view has been confirmed by the results of Tortchinskaya [32], who found that in response to 2.4-D the leaves of *Lupinus albus* and *Nicotiana rustica* accumulate considerable quantities of ammonia.

The P^{32} tracer studies yielded interesting evidence as to the effect of auxonherbicid on phosphorus metabolism. Such data in the literature relate exclusively to results obtained in field trials, in which phenoxyacetic acid was administered in the form of spray, or through the soil and therefore the concentration values were hardly estimable. Our investigations have the advantage of other herbicidal-sensitivity and resistance experiments in that we could neglect differences due to the location and organ differentiations [30, 1, 8, 9], as well as to differences in the rate of translocation within the organism [31, 7, 14] and we could concentrate throughout on the so-called *plasmatic (cytoplasmatic) resistance,* on the determination of the tolerance shown by a given kind of cell [14, 15].

Several pieces of evidence are already available as to the resistance of genetical and environmental factors to herbicidal agents. Such a datum is the difference of a whole order of magnitude in resistance between the monocotyledonous and dicotyledonous plants, in favour of the former [4]. Differences within the same species or organism were demonstrated by Boysen-Jensen [5] in the case of oats, by B. Vargha [6] in the case of peas, by Berezovski [3] and Kovács [24] in the case of maize. The dependence on the grade of nutrition was studied by Wolf [35], and that on the grade of development by Olson [27].

In our experimental object, the potato, *very marked differences could be demonstrated between the various varieties of the plant* that could be measured on the in vitro growth of the tuber tissues (Fig. 2).

The circumstance that the herbicidal effect of 2.4-D and the resistance to that effect, respectively, might be correlated with the respiratory apparatus, has been substantiated by the tomato spraying experiments of Rakitin [30].

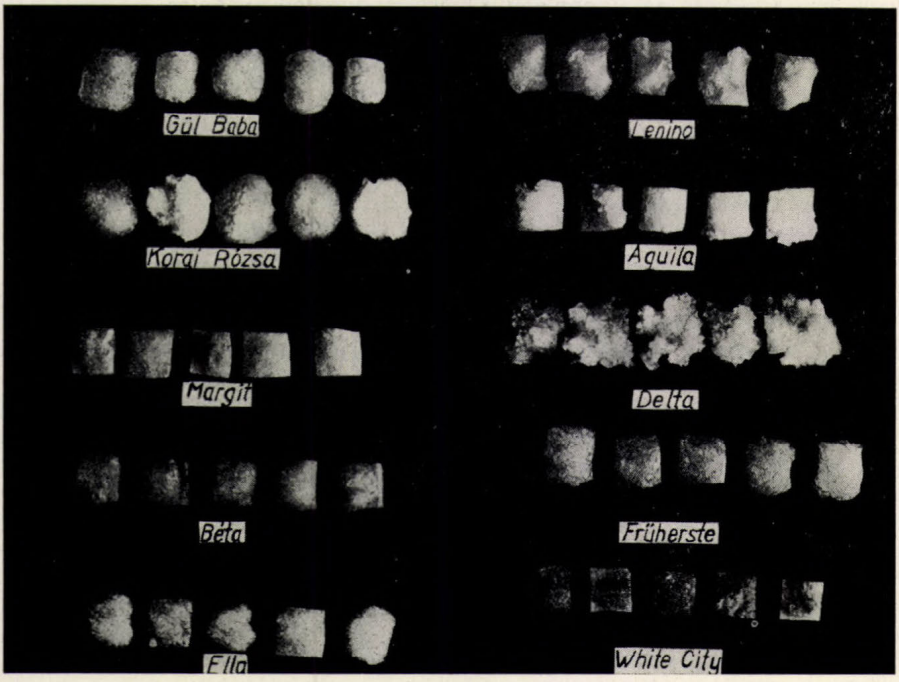

Fig. 2. Varietal differences in the auxonic effect of 2.4-D on potato tissue cultures (10^{-6} M 2.4-D, 14-day culture, original size)

This has encouraged us to concentrate on phosphorus metabolism in the next step of our investigations.

As to the total P concentration, we have found that the auxonic concentration of 2.4-D retards the uptake and accumulation of P, whereas the herbicidal concentration causes a very rapid depletion of the P contents right away. As our earlier experiments [12] showed that 2.4-D did not modify the water contents of the tissues at toxic concentrations, either, and since in this series a certain decrease of total P contents has occurred even with the optimal auxonic 2.4-D concentration, we have no reason to believe that a dilution took place, on the contrary, we should take into consideration that phosphorus might have diffused out into the medium (Table 2). In field experiments a similar decrease of total P was found by Berezovski [3], too.

A comparison of the relative proportions of inorganic and organic P revealed, that in response to the toxic dose of 2.4-D, especially the inorganic P is eliminated at a rapid rate. This could be demonstrated exclusively in the

tissue cultures, in which the P disconnecting from metabolism can readily diffuse out into the environment. The specific activities also indicated that in spite of this loss the specific activity falling to inorganic P was still high. This, on the other hand, clearly shows that this loss can be ascribed to the P fractions diffusing out into the medium.

The difference in behaviour between inorganic and organic P suggested that dichlorophenoxyacetic acid exerted its action in a differentiated way on each P fraction. We should like to point out a few data relative to the influence exerted on the intracellular fate of the P taken from the medium (Fig. 3).

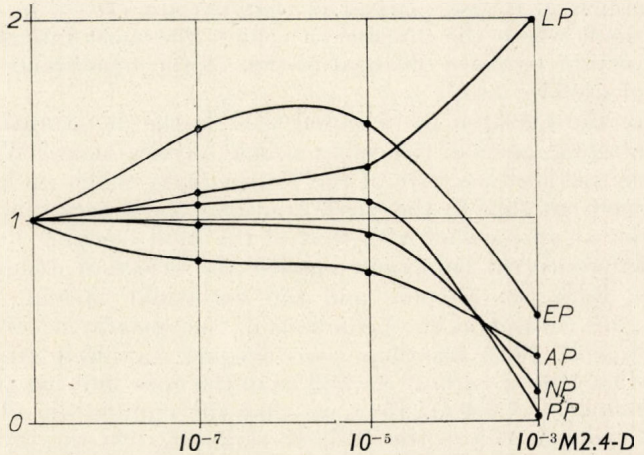

Fig. 3. Effect of 2.4-D concentration on the rate of P uptake by pieces of potato tissue, as related to that by the corresponding fractions of pieces of tissue not containing 2.4-D. (*LP* — lipid-P, *EP* — ethanol-soluble-P, *AP* — acid-soluble-P, *NP* — nucleic acid-P, *PP* — protein-P)

1. The auxonic (growth-promoting) concentration of 2.4-D accelerates its incorporation into the alcohol-soluble and protein fractions, whereas the herbicidal concentration definitely reduces that rate.

2. The rate of incorporation into acid-soluble, high-energy phosphorus compounds is slower in all 2.4-D concentrations than it is in the phenoxyacetic acid-free control.

3. The rate of incorporation into nucleic acid is not modified by phenoxyacetic acid in the well-growing tissues, but it is inhibited at toxic concentration.

4. The rate of incorporation into nucleic acid is inhibited at toxic concentration, otherwise it remains unchanged.

5. It is particularly conspicuous how the incorporation into the lipid-soluble P is modified. In this fraction uptake increases almost linearly at the auxonic concentration, whereas at the toxic concentration it increases in leaps.

It has been deemed important to clarify as to whether the increased incorporation takes place right from the medium, or else: do other phosphorus fractions give their P rapidly away to the compound in the lipid fraction? There is no evidence in the literature pertaining to this point.

To decide the issue, we have set up experimental series in which tissues kept for 6 hours in labelled media were transferred to non-labelled ones. We had

good reason to believe that the specific activity of the fractions deriving P directly from the medium would decrease in a far greater measure at the 12 and 24-hour samplings, than would that of the fractions increasing their P contents by getting P from other intracellular fractions (Table 3).

The results indicate that at the 2.4-D concentrations of 10^{-5} and 10^{-3} M *the specific activity of the lipid fraction,* as related to that of the other fractions, *significantly decreases.* Thus, this fraction gets its very high P contents *directly from the medium in a decisive degree.* And from this we may conclude that one of the sites of action, probably the principal one, of phenoxyacetic acid may be sought for in the *membranes containing lipidphosphorus.* The circumstance that this phenomenon is also marked in that variant (10^{-5}), in which tissue growth is optimal, where the division of cells is the most intense, suggested to us that a certain measure of denaturation of the membranes might be a precondition of growth, too.

After this the question to be posed was: Is the denaturating effect on the lipid-P containing parts of the cells a simple physical noxa? When we were following up the intracellular fate of the P previously taken up by the tracer method, it turned out that in the single fractions the decrease in the specific activity was not at all parallel with that of the lipid fraction, but is showed a definitely differentiated behaviour against the effect of dichlorophenoxyacetic acid, in both the auxonic and the herbicidal variant. Particularly interesting, in this respect, is the behaviour of the specific activity of nucleic acid- and protein-P. Both fractions soon become stabilized after an initial increase in the 2.4-D-free variant, as well as in the ones showing growth under optimal and suboptimal 2.4-D effect, so that the equilibrium of intake and output already sets in before the 12-hour sampling. On the other hand, in response to the toxic concentration of 2.4-D the quantities of nucleic acid and protein P decrease very rapidly. Thus, in that case no dynamic equilibrium sets in.

Our experiments in the field of phosphorus metabolism indicate that the auxonic and herbicidal effects of dichlorophenoxyacetic acid substantially modify P metabolism, and within this in the first place it affects the phospholipids. Phospholipids are known to play a very important role in the building up of permeable cell membranes, and not least of all in that of mitochondria. It seems also plausible that the increase of cellular respiration induced by phenoxyacetic acid also would exert a direct influence on the respiratory apparatus through the altered phospholipid structure of mitochondria. We intend to make an attempt to clarify this problem in future studies involving the use of isolated mitochondria.

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Contributory Discussion

Prof. Romhányi: In connection with the discussed disorders of phosphorus metabolism I want you to remember that the most vulnerable point of the cell is the metabolism of lipids, as its maintenance requires the highest amount of energy. Even slight disturbances of oxidative phosphorylation lead to fatty degeneration. I should like to know whether this toxic or pretoxic effect is morphologically manifested. I should furthermore like to know whether there exist data regarding oxidative phosphorylation, and I should welcome information about the ratio between the macroerg phosphates that arise from inorganic phosphates and the extent of oxidation (P/O). Data published in the course of this symposium are highly interesting from a cytological angle: there are exceedingly sensitive structures in all membranes, and any injury to them affects lipids in the first place. I, too, have found that lipoid components play a very important part. Even the staining of the endoplasmic reticulum or the ergastoplasm depends on the presence of certain lipoid components. The essential function of plasma membranes consists in the specific adsorption of components among which lipids play, on account of their physicochemical properties, a decisive and regulative role. It is well known how important copper is for the metabolism of phospholipids, and that the synthesis of the latter comes to a standstill with possible grave cerebral demyelination in animals kept on diet poor in copper. Does lack of copper provoke disturbances in the lipid metabolism of plants?

Prof. Kellner: The problem we have to decide is whether one is essentially dealing here with a change in the membrane or the accumulation of phosphor containing lipoproteins. Apart from percentual changes I should like to receive information about absolute values. Changes in the amount of phosphorus bound to lipids may indicate whether we have to do with processes concerning the membrane alone or with genuine metabolic changes. Similar investigations are in progress in our institute, although with quite other ends in view. Studying the action of chemotherapeutic agents on the lipoproteins and phospholipids of tumours we observed serious changes only in the fatty acids of the phospholipids, especially in twice or three-times unsaturated fatty acids. It might be worth while to examine the polarometric behaviour of these lipids. We have repeatedly observed a phenomenon often demonstrated in experiments, namely, that there appears a sudden immensely toxic effect above a certain concentration.

Prof. Faludi: Differences between animals and plants have, of course, to be taken into account, but so must the difference between the respective degrees of development. We have made no direct investigations concerning oxidative phosphorylation, and so we have to base our conclusions on the behaviour of the fractions. Inference may further be drawn from rapid and optimal growth which points to normal oxidative phosphorylation. It is the intention to devise still more precise methods of measurement. We want to work on isolated cell fractions. Examinations of this kind that would extend to the whole of the cell are technically almost impossible. The effect of copper on lipids has not been studied in our experiments, but Faludi and Dániel observed a noteworthy effect in carotenoid synthesis. Our medium was such as to exclude the possibility of lack of copper. We have never heard of fatty degeneration in plants. Lipids contained but a few per cents of total phosphorus. A very sensitive method, including the use of isotopes, was necessary for us to be able to obtain reliable data and make accurate conclusions. The literature contains reports on the supposed role of unsaturated fatty acids in plants, especially in connection with photosynthesis, but such reports are somewhat contradictory. I trust that the study of birefringence will prove useful in our work. Our investigations have not yet sufficiently progressed to justify a more exhaustive reply on my part, but the comments advanced here are a great help to us.

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