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Formation of an Atypical Collagen and Cartilage Pattern in Limb Bud Cultures by Highly Sulfated GAG

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Summary. Addition of 1 mg/ml or higher doses of the highly sulfated pentosanpolysulfoester SP54® or the mucopolysaccharidepolysulfoester Arteparon® to limb bud cultures from 11-day-old mouse embryos caused a marked reduction in the growth of the distal parts of the cartilage anlagen. The most striking effect, however, was the change in the collagen structure of the cartilaginous intercellular substance. After more than 0.05 mg/ml SP54® or Arteparon® no collagen filaments were seen but collagen aggregates with an altered cross-striation occurred. They were produced by an antiparallel arrangement of collagen molecules caused by the highly sulfated substances. By immunofluorescence microscopy it was shown that SP54® and Arteparon® did not influence the distribution of the collagen types but only affected the aggregation of collagen type II. From the morphological point of view the production of endogenous PG seemed to be uneffected by SP54® and Arteparon®.

The effect of SP54® and Arteparon® was reversible. After removal of these substances characteristic collagen filaments re-formed. The collagen aggregates were decomposed extracellularly or phagocytosed by chondroblasts and decomposed intracellularly.

Key words: Limb bud cultures (mouse) – Electron microscopy – Effect of highly sulfated GAG (SP54® and Arteparon®) – Collagen structure – Cartilage pattern.

Introduction

Proteoglycans (PG) and glycosaminoglycans (GAG) have numerous biological functions (Ogston, 1970; Kimmig and Kreysel, 1973; Scott, 1975). During mor-

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phogenesis they e.g. influence proliferation, differentiation, and migration processes (Lippman, 1968; Montagnier, 1971; Rubin, 1971; Toole, 1973; Toole and Gross, 1971; Toole et al., 1972; Nevo and Dorfman, 1972; Bernfield et al., 1973; Kimata et al., 1973; Kosher et al., 1973; Hay and Meier, 1974; Huang, 1974; Meier and Hay, 1974; Karp and Solursh, 1974; Kosher and Lash, 1975; Meier and Hay, 1975; Pratt et al., 1975; Slavkin and Greulich, 1975; Spooner and Conrad, 1975; Strudel, 1975, 1976; Wiebkin and Muir, 1975; Chiarugi and Vannuchi, 1976; Kosher, 1976; Reddi and Anderson, 1976; Schaffrath et al., 1976; Clowes and Karnowsky, 1977; Coupar and Chesterton, 1977; Seki and Oda, 1977).

An other function of PG and GAG is based on their binding to collagen, which influences its aggregation during fibrillogenesis. In in vitro experiments it has been shown that PG and GAG are able to stimulate or inhibit the nucleation of tropocollagen molecules and thickness growth of fibrils (Jackson, 1953; Wood, 1960; Keech, 1961; Mathews, 1965; Mathews and Decker, 1968; Toole and Lowther, 1968; Chapman and Armitage, 1972; Lowther and Natarajan, 1972; Gelman and Blackwell, 1973; Öbrink, 1973a, b; Nemeth-Csoka, 1974; Ananthanarayanan and Nimni, 1975; Öbrink et al., 1975; Oegema et al., 1975; Franzblau et al., 1976; Toole, 1969, 1976; Anderson et al., 1977). The cross-striation pattern can also be changed by these substances (Kühn, 1962; Kühn and Zimmer, 1961; Kühn et al., 1959, 1964). Contradictory results in such experiments are possibly due to variations in the collagen preparations used, or to the different time of application of PG and GAG during the phase of fibrillogenesis.

Experimental treatment of these problems in vivo is impeded by the poor permeability of highly charged PG and GAG. The same it true of the investigations on the effects of PG and GAG on mammalian embryos with a placental barrier. These difficulties can be avoided by using limb bud cultures into which PG and GAG penetrate (Merker et al., 1977). In limb bud cultures of day 11 and 12 mouse embryos large amounts of collagen type I and II are produced (Barrach et al., 1975; Neubert et al., 1974) and from undifferentiated blastema a cartilage skeleton develops whose pattern largely corresponds to the in vivo picture (Merker, 1975). This model seems therefore to be suitable for the investigation of the influence of exogenically applied PG and GAG on the aggregation of collagen and the proliferation and differentiation of the cartilage anlagen.

Preliminary studies have shown that the aggregation pattern of collagen is affected by the pentosanpolysulfoester SP54® (Merker et al., 1977). The aim of the present study is to investigate the structure of collagen after application of SP54®, the dose-response relationship, the dependence of the duration of action, the effect of growth, differentiation and pattern formation of the cartilage skeleton in vitro and the reversibility of these effects. Another substance producing similar effects is the mucopolysaccharidepolysulfoester Arteparon®, which has been used for comparison.

Materials and Methods

Mouse embryos (strain NMRI) on gestation day 11 (42 to 45 somite pairs) were used. The upper limb buds were removed under sterile conditions and stored in HANK-BSS-medium (Aydelotte

and Kochhar, 1972; Neubert et al., 1974; Merker, 1975). The culture medium consisted of BGJ (Biggers et al., 1961) plus 25% fetal calf serum (Biocult Lab.) and 150 µg/ml L-ascorbic acid (MERCK). The limb buds were cultured in a Trowell-system (Trowell, 1959). For this purpose they were placed on a membrane filter consisting of cellulose nitrate (Sartorius, pore width 12 µm) over an B-4A stainless steel grid. The gas phase in a gas-supplied incubator consisted of moistened air plus 5% carbon dioxide. The medium was changed every 3 days.

The substances SP54® (pentosanpolysulfoester, molecular weight 2,000 daltons), 3,8 SO_4/disaccharide; Benechemie, München) and Arteparon® (mucopolysaccharidepolysulfoester, molecular weight 12,000 daltons, Luitpold-Werke, München) were added in the first experimental series on day 1 or 2 in vitro and again on day 3 when the medium was changed. In the following series SP54® or Arteparon® were added only on days 1 to 3 or 4 to 6. The concentrations of SP54® and Arteparon® amounted to 0.001; 0.005; 0.01; 0.05; 0.1; 0.5; 1.7; 3 or 4 mg/ml culture medium. The experimental groups and the control group without GAG consisted of 16 limb buds each.

For the morphological investigations the limb buds were fixed on days 3,4,5, or 6 in a solution which contained 3% paraformaldehyde, 3% glutaraldehyde in 0.2 M cacodylate buffer pH 7.2 and 0.5% ruthenium red. Postfixation was performed in 1% OsO_4 in cacodylate buffer pH 7.2 with 0.5% ruthenium red. This was followed by dehydration in an acetone series and embedding in Mikropal. Sectioning was carried out in LKB- and Reichert microtomes and contrasting was performed with uranyl acetate/lead citrate. Electron microscopy utilised a Zeiss EM10 and a Siemens Elmiskop 101. For light microscopical investigations thicker sections (<1 µm) were stained with alkaline Giemsa solution at 60° C for 1 min.

For the fluorescence microscopical demonstration of the various collagen types with collagen antibodies 8 µm thick sections from unfixed limb buds after a 6 days culture period were cut in a cryostat, mounted onto slides and covered with antibodies against collagen type I and II (50–100 µg/ml).

The collagen antibodies were obtained by repeated immunisation of sheep and rabbits with purified collagen type I or II and Freund's adjuvant and were purified by immunoabsorption. Their specificity was tested in a passive hemagglutination test (Beil et al., 1972; Barrach et al., 1975). The collagen types were demonstrated by reacting the antibodies with fluorescein conjugated goat anti-rabbit gamma-globulin (Behring-Werke, Marburg) at a dilution of 1:10.

For the determination of the cartilaginous parts the limb buds were fixed in 10% buffered formaldehyde, stained with methanolic methylene blue, destained with HCl-methanol., dehydrated and cleared with glycerol and covered with Eukitt. Planimetrical measurement of the cartilage areas was performed automatically in a Quantimet 720 system (Metal Research).

Results

Light Microscopy

On day 6 of the culture period the proximal skeleton anlagen show the characteristic picture of embryonic cartilage with a well-defined perichondrium. In the diaphyseal region a delicate osteoid rim can often be demonstrated between cartilage and perichondrium. The cells of the distal cartilage anlagen are more closely apposed due to the small amount of intercellular substance present. A clear-cut outline of a perichondrium cannot be seen. Addition of SP54® or Arteparon® does not cause any changes.

Demonstration of the Cartilage Anlagen

After a 6 day culture period all cartilage anlagen stainable with methylene blue amount to 1.0 ± 0.04 mm^2/limb bud ($=100\%$). This value does not change after lower doses of SP54® or Arteparon® (0.001–0.05 mg/ml). It increases slightly after 0.1 mg/ml, however, without becoming statistically significant. With

1.0 mg/ml and higher SP54® or Arteparon® doses the planimetrically measured areas of cartilage anlagen are reduced to 70% (1 mg/ml), 62% (2 mg/ml) and 55% (3 mg/ml).

It is principally the distal cartilage anlagen that are affected by a reduction in area (Fig. 1). After 4 mg/ml SP54® the total cartilage skeleton of the hand region is missing in all cases. After 1.7, 2 and 3 mg/ml the number of rays of the hand skeleton is diminished. After 1.7 to 3 mg/ml SP54® often only a single ray occurs, with no segmentation into carpus, metacarpus, or phalanges. The frequent occurrence of a half-moon-shaped cartilage sickle located distally to the single ray is a striking feature. Such diffuse distal areas of cartilage are never found in control cultures.

Immunofluorescence Microscopy

In control limb buds antibodies against collagen type II can only be demonstrated in cartilage. When using anti-collagen type I fluorescence is localized over the perichondrium and, to a lesser extent, over other regions outside the cartilage. Differences between SP54®- or Arteparon®-treated and control limb buds were not observed (Fig. 2).

Electron Microscopy

After a 6 day culture period the chondroblasts of the cartilage anlagen are fully differentiated. Their shape is mainly round to polygonal but occasionally elongated or half-moon-shaped. Small cuspidal processes of varying length are characteristic. Cavities of rough endoplasmic reticulum predominate in the cytoplasm. The Golgi apparatus occupies a large space in the vicinity of the nucleus and consists of some short, parallel cavities and several vacuoles of 1,000–4,000 Å in diameter. The centres of these cavities contain loosely packed fine-filamentous material. Moreover, a few mitochondria of the cristae type are found as well as single electron-dense granules. In many preparations the chondroblasts of the diaphyseal region store glycogen granules which fill large areas.

The intercellular space is filled by collagen structures and ruthenium-red (RR)-positive proteoglycan granules (Fig. 3). Collagen occurs in the form of loosely packed, single, irregular filaments of 80–160 Å in diameter. Only in very large intercellular spaces are 3 to 5 filaments occasionally seen to run parallel forming small bundles. Round or rectangular ruthenium-red-positive electron-dense granules (350–550 Å) rest on the filaments at irregular intervals and from these granules fine threads of 20–50 Å thickness radiate. A great number of such granules are also situated between the collagen filaments. Their radiating fine threads make contact with the collagen filaments and disappear from the section plane or link with the neighbouring granules or their threads. In most preparations the granules in the vicinity of the cells are so closely packed that they cover the delicate collagen filaments.

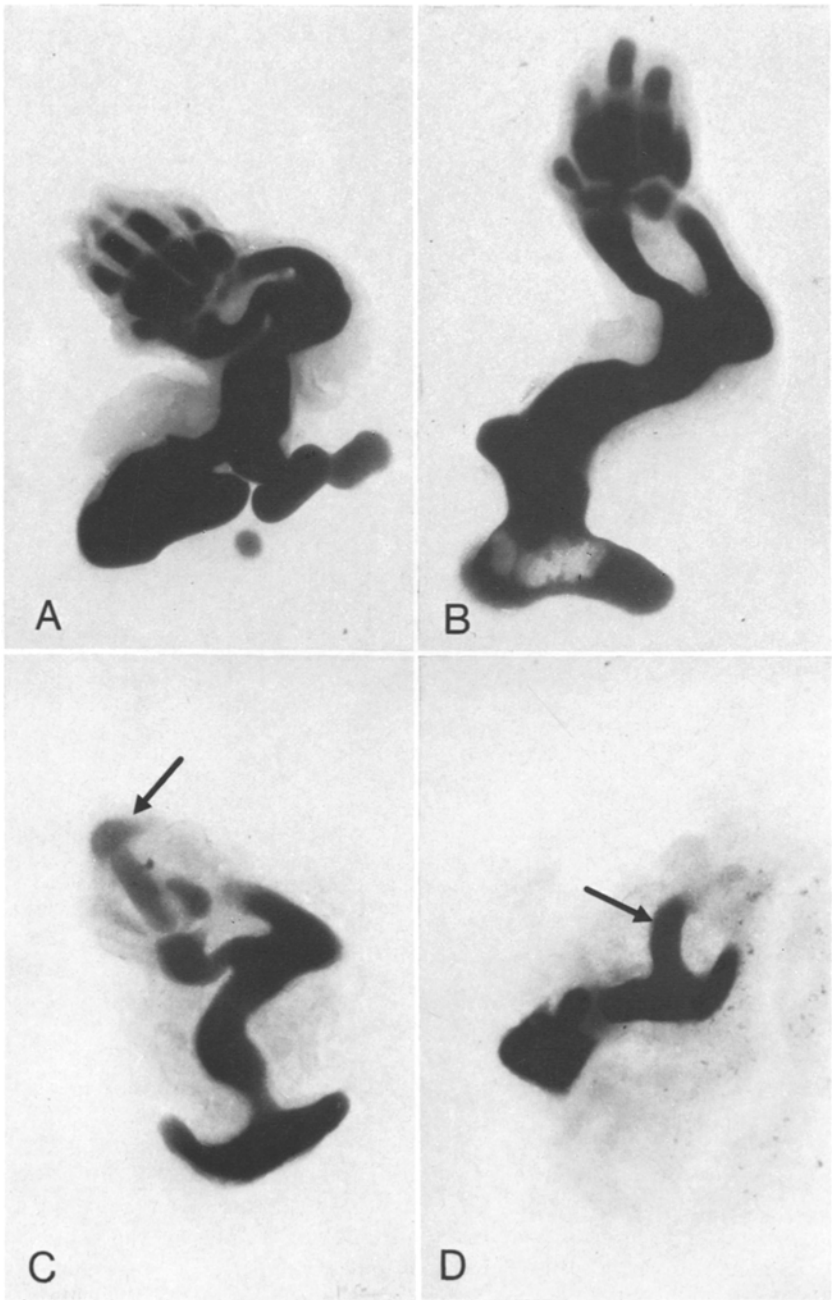

Fig. 1A–D: Limb buds stained with methylene blue and cleared in xylol after a 6 days culture period. **A** Control. **B** Treated with 0.1 mg/ml SP54®. **C** Treated with 2 mg/ml SP54®. Absence of tarsalia and metatarsalia, sickle-shaped cartilage region (↓) distally closely beneath the epithelium. **D** Treated with 4 mg/ml SP54®, complete lack of the distal regions, only humerus, radius and ulna (↓) demonstrable

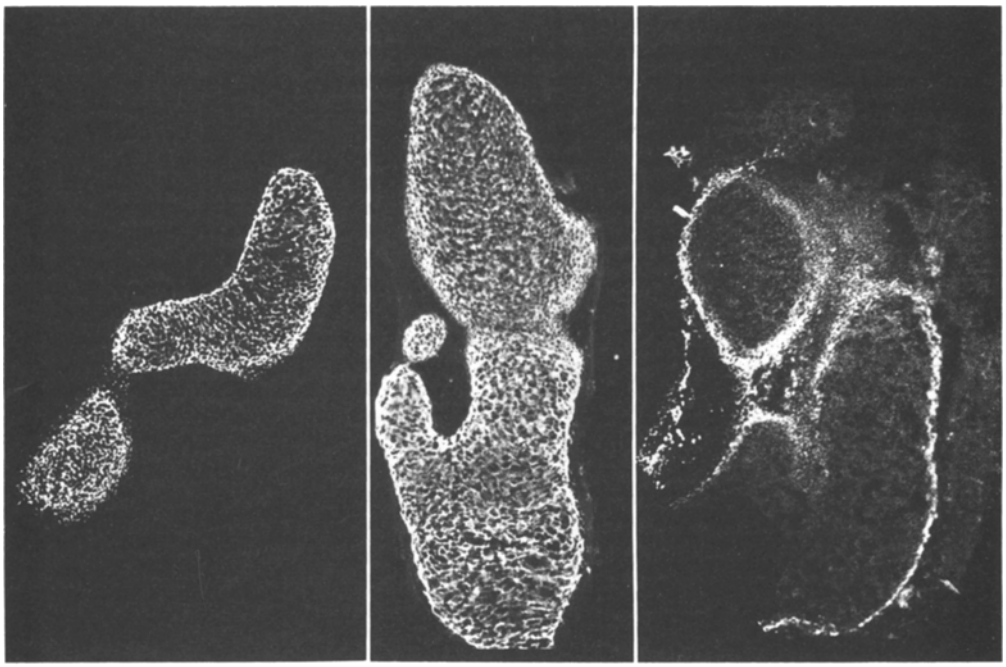

Fig. 2 A–C. Immunofluorescence microscopical demonstration of the various collagen types in cultured limb buds. **A** Collagen type II in the cartilage tissue of an untreated limb bud. **B** Collagen type II in the cartilage tissue after treatment with 3.0 mg/ml SP54®. **C** Collagen type I in the perichondrium and in the tissue outside the cartilage after treatment with 3.0 mg/ml SP54®

Even after very high doses of SP54® and Arteparon® applied for 5 and 6 days respectively the fine structure of chondroblasts is not changed on day 6 in vitro, only in a few sections have the number and size of the glycogen inclusions in the cartilage cells increased. In the distal region, however, cartilage formation is low or missing completely after exposure to 4 mg/ml.

However, the collagen of the cartilage anlage shows a completely different structure in the presence of more than 0.05 mg SP54® or Arteparon® per ml culture medium. Short aggregates of varying length occur which display a defined periodic cross-striation (Fig. 4), the length of the shortest aggregates is 2,800-3,100 Å (monomeric aggregates). The length of one period in the longer aggregates amounts to 2,000-2,700 Å. These structures can be called "fibril long spacing (FLS)-collagen". They first occur a few hours after addition of SP54® or Arteparon®, and increase in number during culture in the presence of these substances. After doses higher than 1 mg SP54® per ml culture medium normal collagen filaments no longer occur in the cartilage anlage. Outside the FLS-aggregates only ruthenium-red-positive granules can be found in the intercellular space.

The cross-striation in the collagen aggregates is symmetrical (Figs. 5, 6, and 9). Two relatively indistinct, electron-dense zones of 250 Å width lying in the centre of each period separated by a less dense zone (280 Å) are striking. At higher magnification up to 6 bands (25 Å each) can be demonstrated in

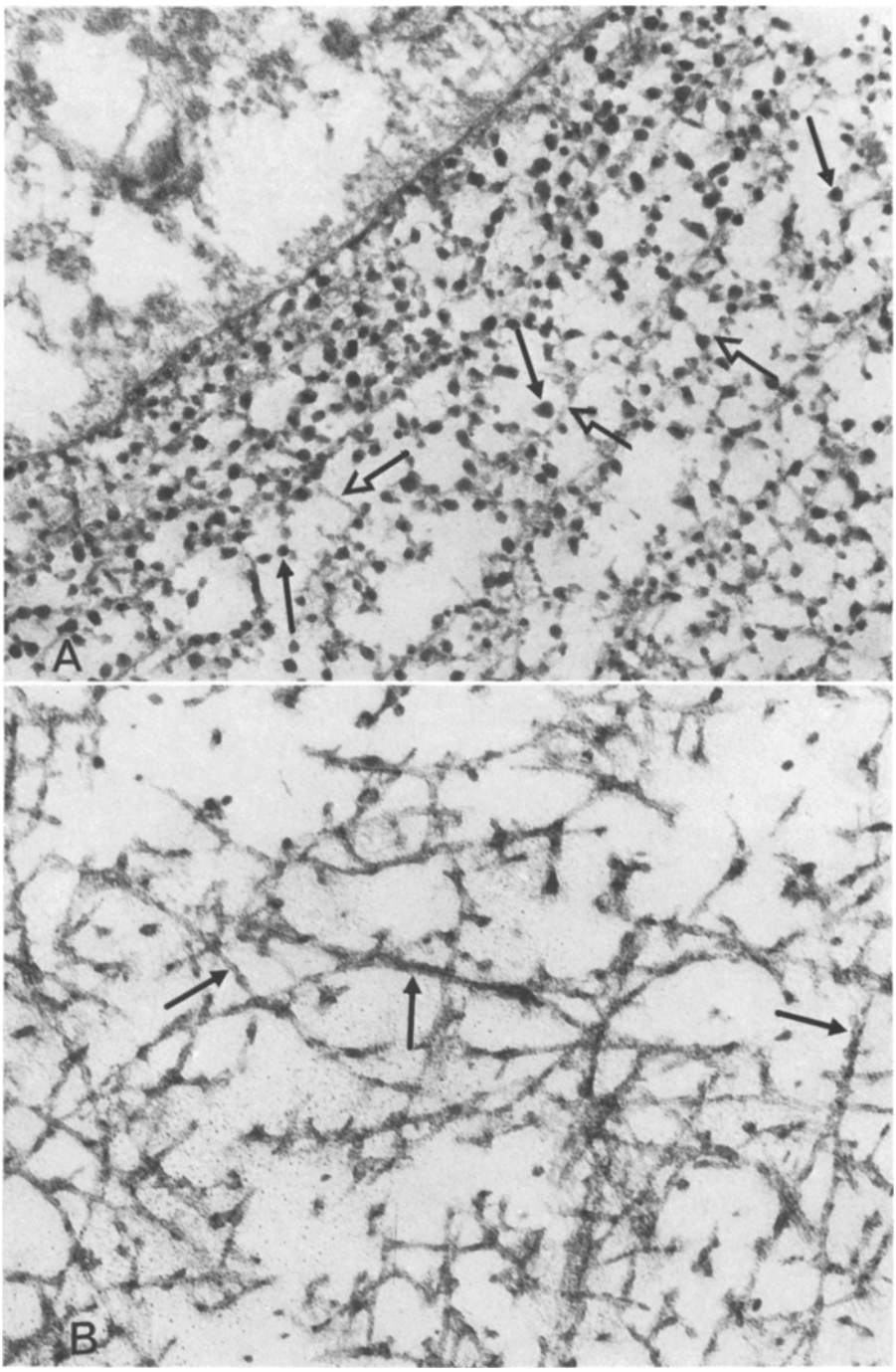

Fig. 3A and B. Cartilage tissue from the humerus anlage of untreated limb buds after a 6 days culture period. **A** Ruthenium-red staining, numerous electron-dense PG-granula (↓) and collagen filaments (light arrows) in the vicinity of a chondroblast (*C*). ×63,000. **B** Without ruthenium-red staining. Demonstration of the collagen filaments (↓). ×75,000

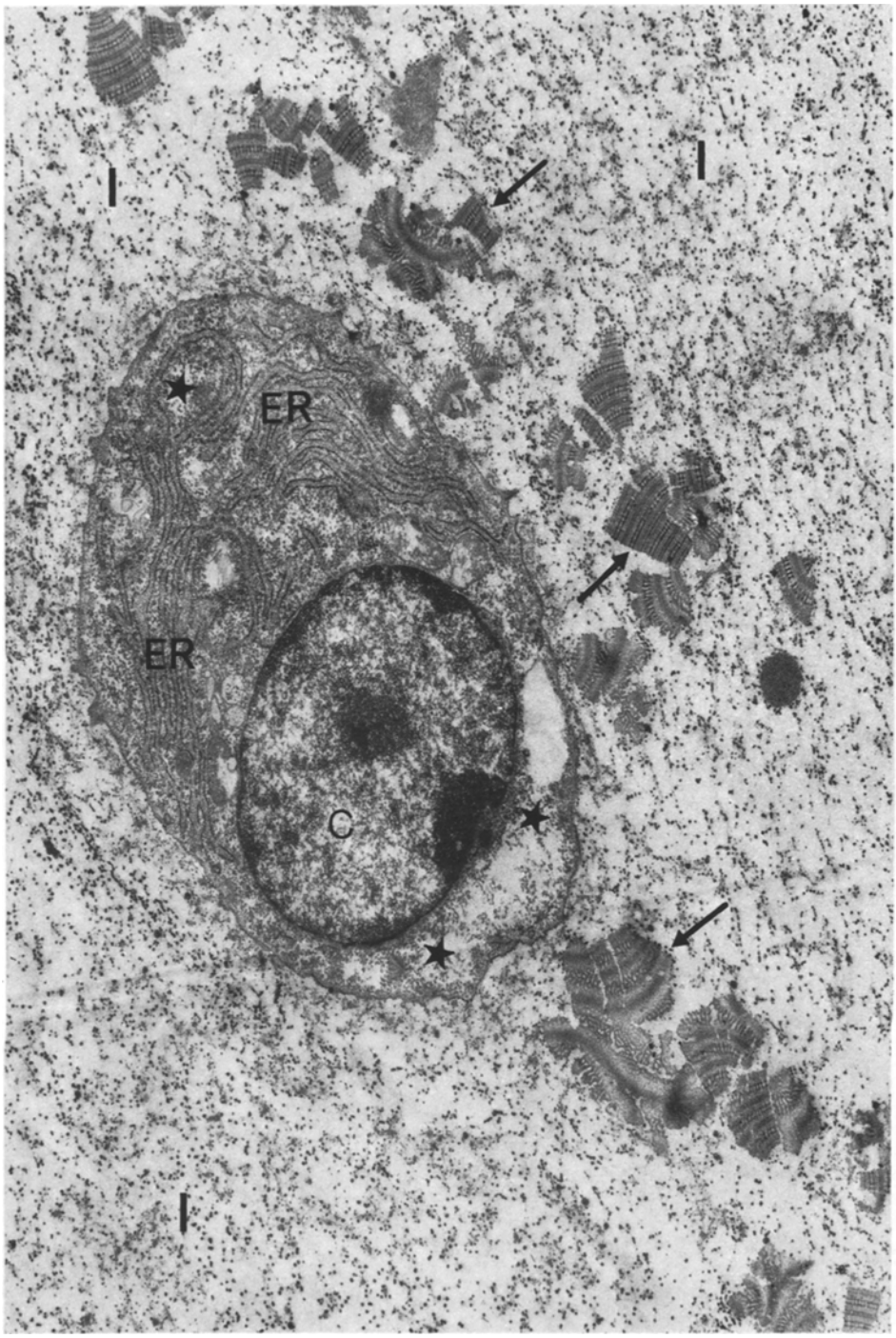

Fig. 4. Limb bud culture after a 6 days culture period. 1.7 mg/ml SP54® added. Chondroblast (*C*) with rough endoplasmic reticulum (ER) and glycogen areas (*). In the intercellular space (*I*) ruthenium-red-positive PG-granules and cross-striated, segment-like structures (↓). ×12,000

each dense zone (Fig. 6). These 6 bands are arranged at about 20 Å intervals. In the lighter zone between the 2 dense zones two clear-cut bands (40 Å each) are running at a distance of 80 Å, enclosing a faint third band. This delicate third band constitutes the symmetrical axis of the cross-striation pattern which is identical on both sides. On each side the dense zones consisting of 6 bands are followed by a band pair at a distance of 30 Å dense line. Adjacent to this band pair are on each side: a light zone of 60 Å, a band triplet (*30* Å – 25 Å – *30* Å – 25 Å – *30* Å), a light zone of 40 Å, another band triplet (*50* Å – 40 Å – *50* Å – 40 Å – *50* Å), a light zone (40 Å), a group of bands (*40* Å – 55 Å – *30* Å – 30 Å – *30* Å – 55 Å – *40* Å), the outside ones being very dense, a light zone (200 Å), a denser band (40 Å), and finally a 200 Å wide lighter zone with a faint denser border. The diffuse density of the double zone in the centre of a monomeric aggregate (250 Å – 280 Å – 250 Å) is notable, as in the bundling of the filamentous subunits. This region, therefore, conveys the impression of longitudinal striation of the aggregates (Figs. 5 and 6). Up to 6 of these monomeric segments can be aggregated end to end, with overlapping ends. Two types of overlapping can be observed, one amounts to 240 Å, the two clear-cut distal bands thus forming a dense double band followed on both sides by the typical triplet. The second type of overlapping is 720 Å in length and a symmetrical triplet pair develops. Both types of overlapping are found in one FLS-aggregate. The width of the aggregates varies considerably and after only 24 h of SP54® and Arteparon® application, widths of 750–1,000 Å can be measured. At later stages values between 0.5 and 1 µm predominate. However, wider aggregates as well as aggregates that arrange themselves circularly can also be observed.

Outside the cartilage collagen type I occurs in the perichondrium, in the muscle anlagen, and in the region of the corium (Fig. 2). In these areas thicker fibrils (250–350 Å) with a clear-cut cross-striation predominate electron microscopically. After SP54® and Arteparon® the appearance of these fibrils does not change under these experimental conditions.

The ruthenium-red-positive, electron-dense granules with radiating filaments do not change after the addition of the two substances. As the normal collagen filaments are missing in many preparations free ruthenium-red-positive granules predominate. In some areas they show a pronounced variability in size (Fig. 7), their diameter varying between 250 and 1,500 Å (normal value = 350–550 Å). The large granules usually rest on a single collagen filament, aggregates or cell membranes. – A regular spatial relationship of the granules to the SP54®- or Arteparon®-conditioned aggregates or their cross-striation cannot be identified.

If SP54® is added for the first 3 days and the culture is then continued without the substance, some characteristic alterations can be observed on the subsequent days. In the cytoplasm of the chondroblasts large vacuoles whose bordering membranes correspond to the structure of the cell membranes occur to an increasing extent (Fig. 8). The 0.4–1 µm large vacuoles contain loosely dispersed fine filamentous material and thin aggregates with cross-striation. RR-positive granules are not seen. Transitional forms between the smaller Golgi-vacuoles and large inclusions could not be clearly demonstrated. The number of vacuoles containing cross-striated structures increases towards the periphery

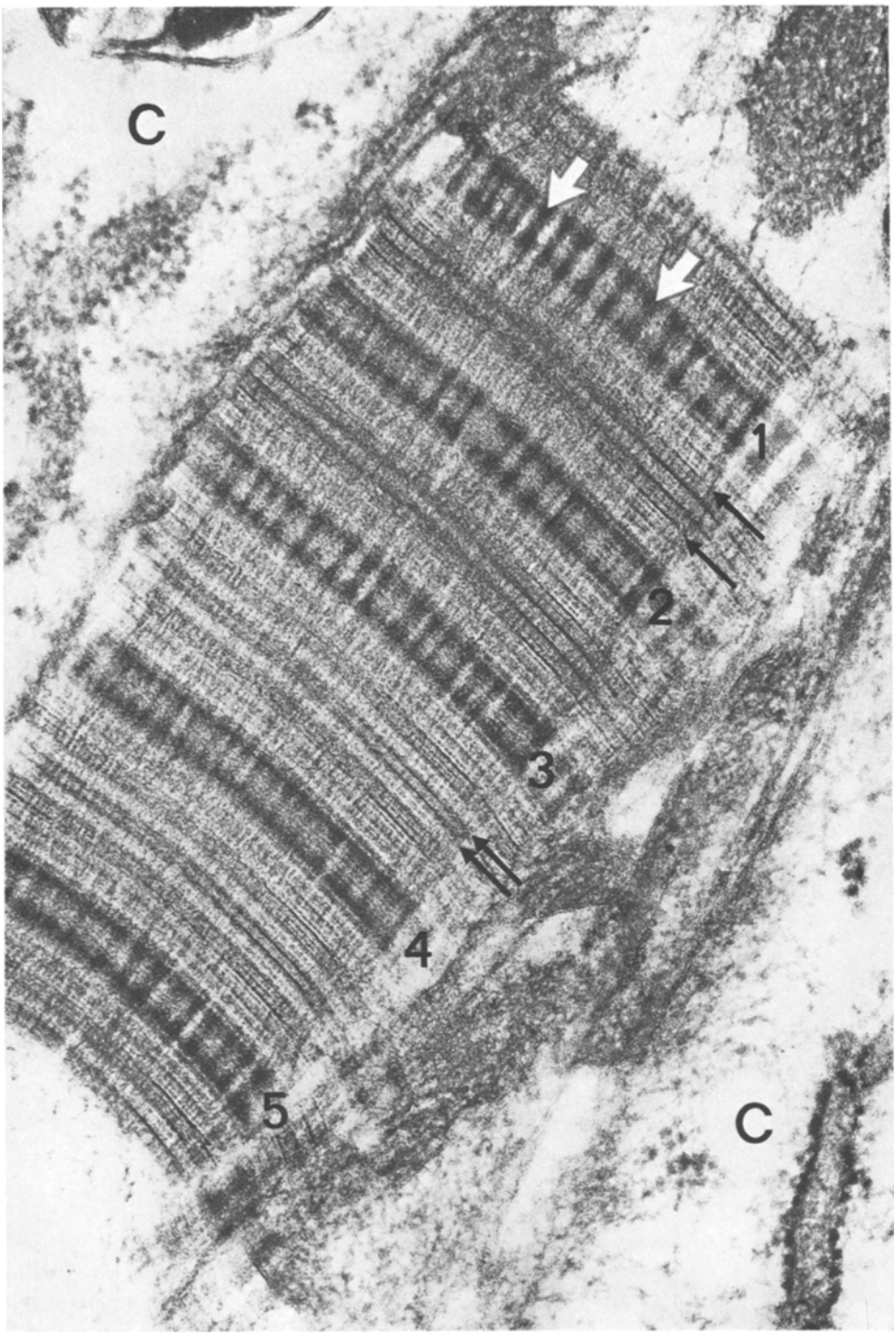

Fig. 5. Limb bud culture after a 6 days culture period. 1.7 mg/ml SP54® added. Cross-striated FLS-structure between chondroblasts (*C*) with 5 successive periods (*1–5*). Between 1 and 2 and 2 and 3 a longer overlapping zone (↓) than between 3 and 4 and 4 and 5. In the centre of each period an electron-dense double band with bundling of the filamentous components at 1–5 (light arrows). × 110,000

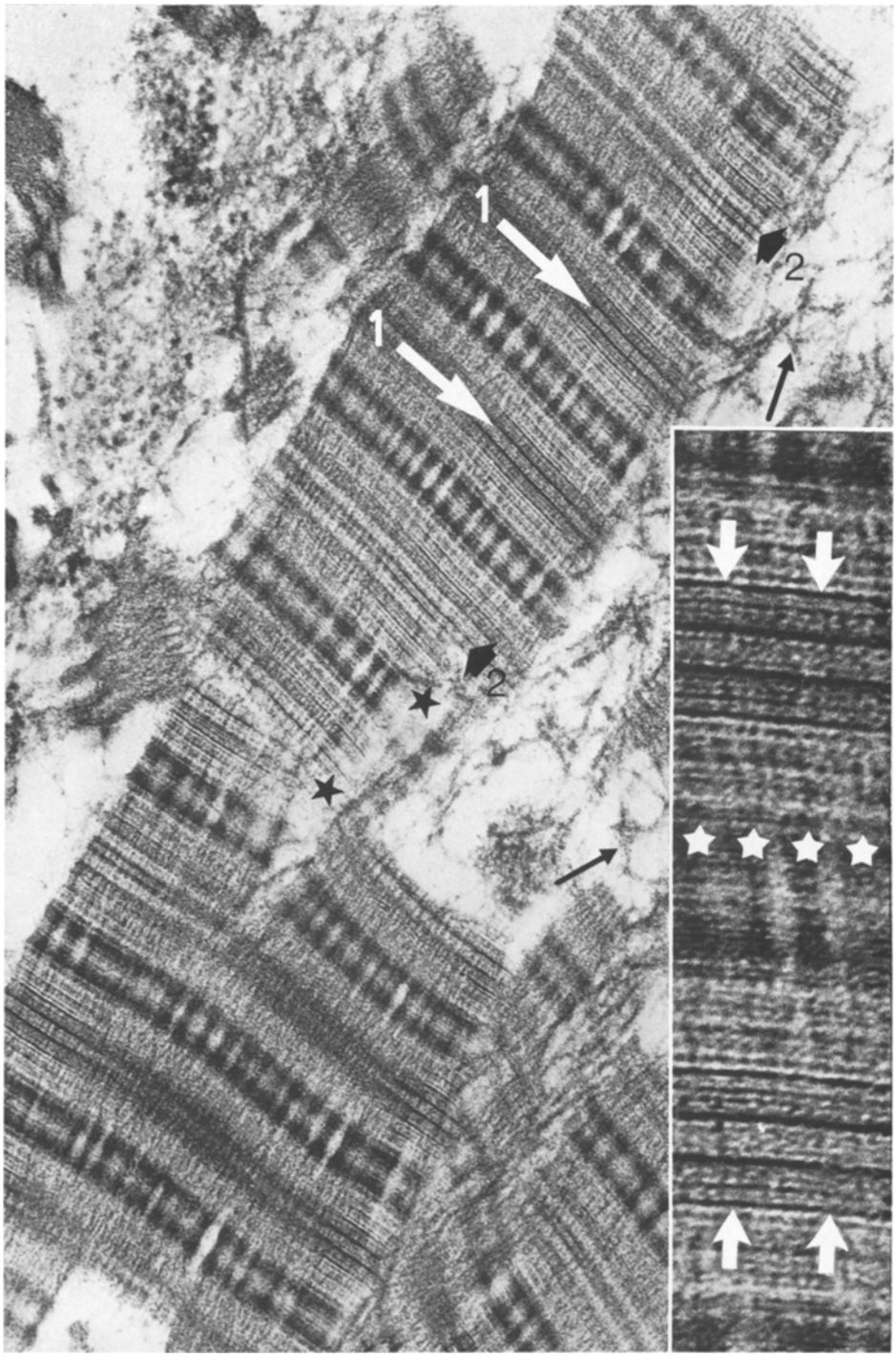

Fig. 6. Limb bud culture after a 4 days culture period. 1.7 mg/ml SP54® added on days 1–3. Overlapping zones of varying width between the individual periods (*1*, light arrow = short, *2*, dark arrow = long overlapping type). At * absence of one period. Single normal collagen filaments (↓). Heavy electron-dense and plat-like bundling in the centre of each period. ×80,000. *Inset:* Cross-striation type of a collagen aggregate. One period between the light arrows, long type of overlapping. Plat-like bundling (light asterisk) in the region of the period centre. ×250,000

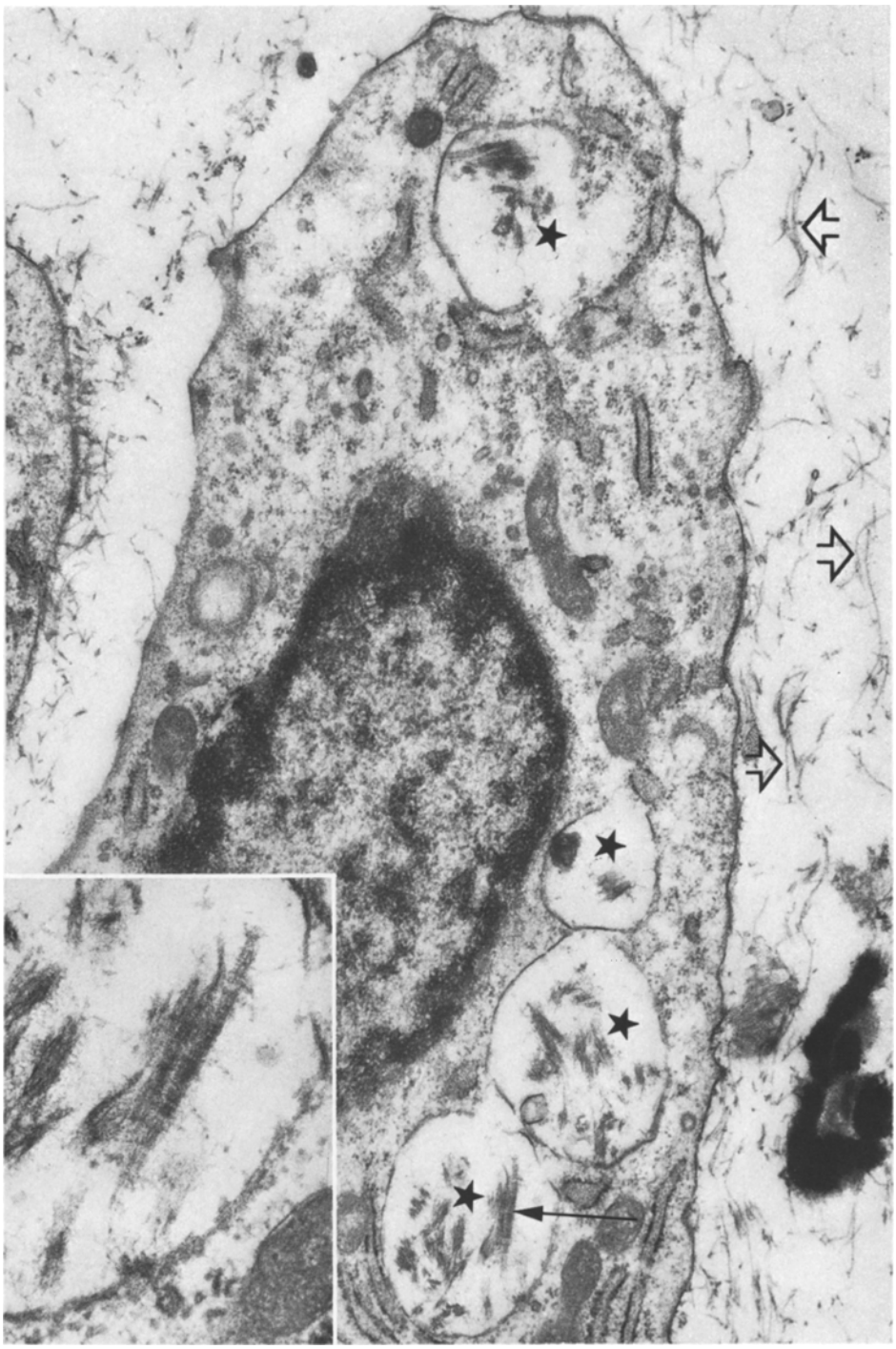

Fig. 7. 6-day-old limb bud culture. 3 mg/ml SP54® added on days 1–3. In a chondroblast vacuoles (*) with filamentous, partly cross-striated (↓) structures are seen. In the intercellular space normal collagen filaments occur (*light arrow*). ×30,000. *Inset:* Higher magnification of the lower inclusion. ×80,000

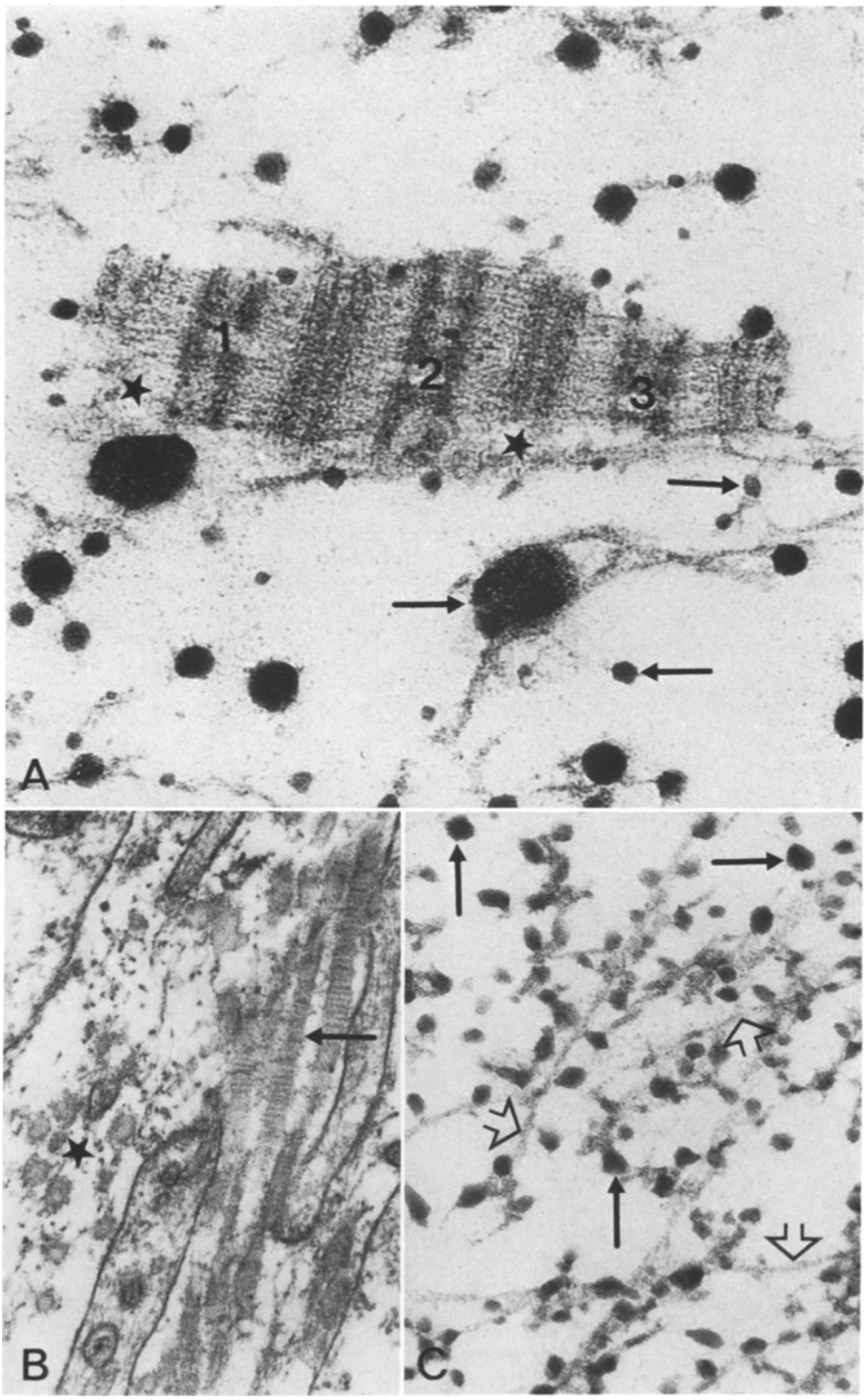

Fig. 8. A 6-day-old limb bud culture. 2 mg/ml SP54® added on days 1–3; three successive periods (1–3) with marked disintegration and melting starting at the ends (*). Widely varying size of the ruthenium-red-positive PG-granules (↓). ×110,000. **B** 6-day-old limb bud culture, 4 mg/ml SP54® added. Perichondral region (collagen type I) with unchanged longitudinal (↓) and cross-sectioned (*) collagen fibrils. ×60,000. **C** 6-day-old limb bud culture, untreated control. Ruthenium-red-positive PG-granules (↓) and collagen filaments (light arrows) in the cartilaginous intercellular space. Compare with **A**. ×90,000

of the cartilage where they are especially frequent in the extracellular space the number of aggregates decreases after removal of the substances from the medium. 3 days after termination of SP54® application only aggregate monomeres (2,800–3,100 Å) or smaller fragments can be observed. Cross-striation largely disappears, only the wide central, dense band pair can still be identified. The fragments reveal disintegration starting from the ends (Fig. 7). Parallel to this the normal 80–160 Å thick collagen filaments reoccur.

Discussion

In the presence of SP54® or Arteparon® unusual collagen structures occur in the cartilage anlagen of limb bud cultures. This striking finding may be due either to the biosynthesis and secretion of a collagen different to type II or to a changed aggregation pattern of the collagen molecules. Although a change in the collagen type synthesized is found under certain conditions in cartilage cultures (v.d. Mark et al., 1977), immunofluorescence investigations revealed the presence of the characteristic collagen type II. Thus, under the influence of SP54® and Arteparon®, procollagen type II is formed which is aggregated into a different pattern. These assumptions are also supported by in vitro experiments with highly purified soluble collagen type II from cartilage tissue (Lilja and Barrach, unpublished findings). During dialysis against SP54® collagen structures are formed which correspond to the picture described here. It can therefore be assumed that SP54® and Arteparon® change the normal aggregation of collagen, possibly due to the strong electrostatic interactions between the highly sulfated substance and the polar groups of the collagen molecule (Kühn et al., 1959; Öbrink et al., 1975; Oegema et al., 1975). Mainly short „fibril long spacing" – collagen structures will develop in which tropocollagen molecules are arranged in an antiparallel manner (Fig. 9). It is known that only under these conditions will the individual monomeric segments show an end-to-end arrangement, thus forming longer units (Kühn et al., 1964). The strictly symmetrical arrangement of the cross-striation pattern also provides corroboration for the assumption of an antiparallel arrangement of polar collagen molecules. At the terminal contact areas the collagen molecules overlap

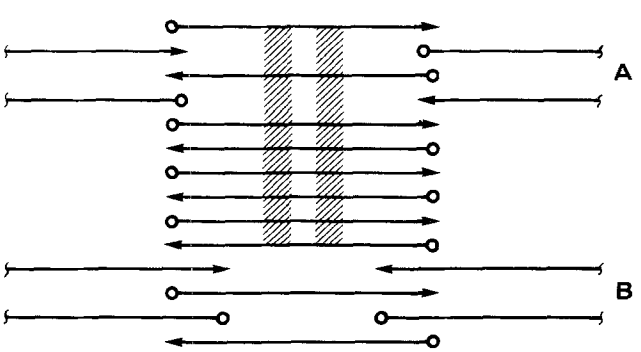

Fig. 9 A and B. Schematic presentation of the antiparallel arrangement of the tropocollagen molecules in a period and the short (**A**) and long (**B**) overlapping zones at the ends

in two ways: long (720 Å) and short (240 Å) values corresponding to about 1/4 or 1/12 of the length of a collagen molecule. Other collagen structures, such as native fibrils and fibrils with a continuous cross-striation also show overlapping of the collagen molecules to a comparable extent (Kühn et al., 1964). The two types of overlapping give rise to a different cross-striation pattern in these contact areas, which may be explained by superimposition of the segment ends of corresponding length (Fig. 10). In this connection findings of Bonucci et al. (1975) are of special interest. In a case of osteopetrosis fetalis these authors were able to demonstrate FLS-collagen in the intercellular substance of rib cartilage. This FLS-collagen was very similar to the structures observed by us. Therefore it seems to be justified to assume an alteration of proteoglycans in this syndrome. According to our findings, the production of anomalous collagen is less likely.

The alteration of the aggregation pattern after application of SP54® refers only to collagen type II. Collagen type I, which occurs outside the cartilage anlage in the limb buds (Barrach et al., 1975), is not affected. The reason for this specificity is not known. Differences in concentration or diffusion problems with these substances cannot play any rôle, since the localisation of the various tissues is very variable and in many preparations the other tissues are close to the filter. Independent of the localisation of the cartilage, FLS-collagen occurs in the limb buds at equivalent concentrations of SP54® or Arteparon®.

SP54® and Arteparon® cannot be demonstrated electron microscopically with certainty. Even after high doses the electron microscopical picture of RR-positive PG-granules in cartilage anlagen of limb buds does not change over wide areas. However, in some places the average size of PG-granules changes. In our opinion this is due to a preparation-conditioned artefact. The PG-molecules are uncoiled under in vivo conditions and fixation and electrostatic binding of ruthenium-red cause clumping of the filamentous molecule which appears as a granule in the electron microscopical picture (Merker and Günther, 1973). Greater change in the three-dimensional PG-pattern during preparation is prevented by the binding of these molecules to the stable network of the collagen structures. This stabilising influence is, however, diminished due to the different aggregation pattern of collagen in the presence of SP54® and Arteparon®. It is conceivable that fixation leads to a clumping of neighbouring PG-granules and thus to an increase in size. Hence, according to our morphological findings, SP54® and Arteparon® do not effect PG-synthesis. The alteration in the collagen structures has to be attributed, therefore, to the added substances (SP54® and Arteparon®) and not to a change in locally produced collagen or PG.

An effect of SP54® and Arteparon® on growth and pattern formation of the cartilage skeleton can be demonstrated. A planimetrically measured decrease in cartilage size might be due to an effect on cartilage cell proliferation as well as on matrix production. Preliminary studies (Lilja) have shown that a dose-dependent reduction in DNA content and corresponding reduction of RNA and protein values occur. This is an indication of inhibition of the proliferation of cartilage cells or their precursors. The inhibition of distal cartilage growth may be attributed to the presence of a proximo-distal maturation gradient. Intercellular substance is produced in the humerus anlage as early as after

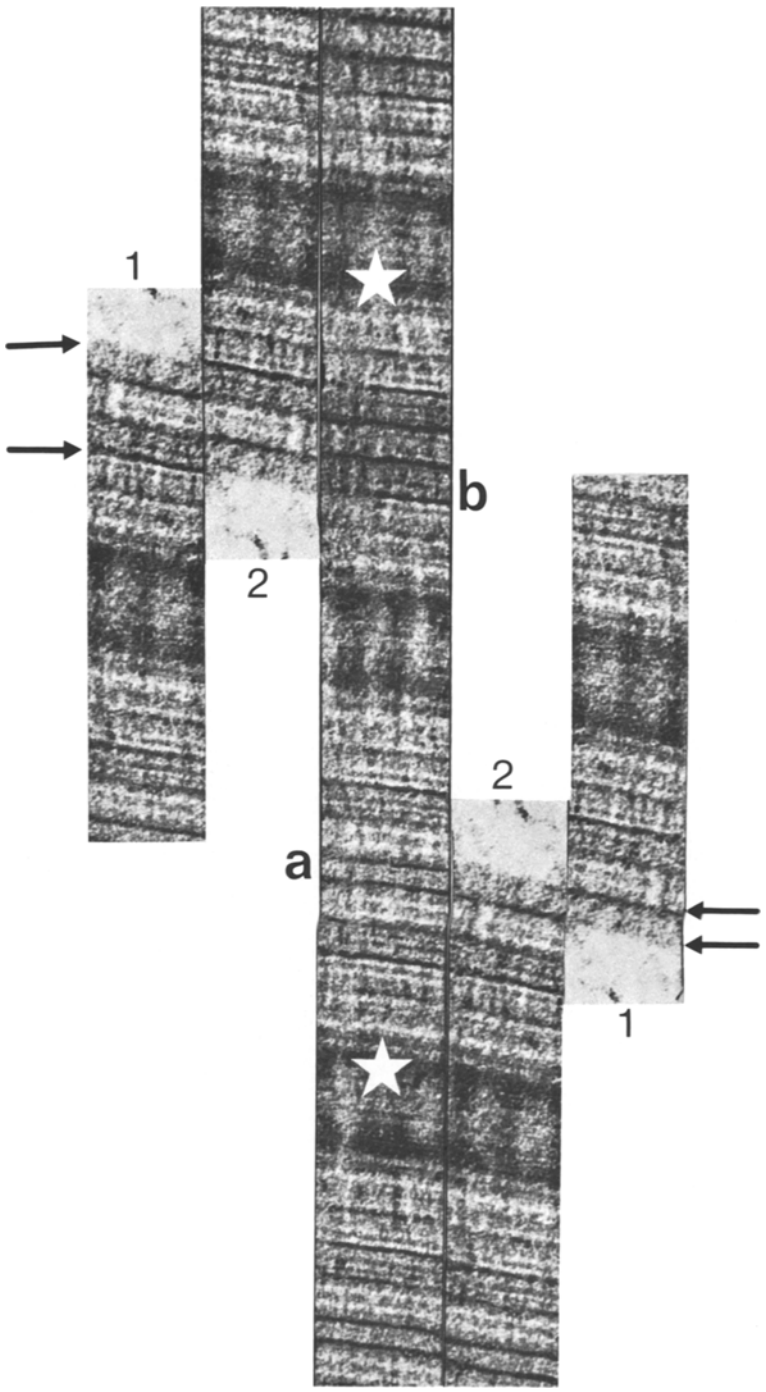

Fig. 10. Photo montage to demonstrate the development of the cross-striation pattern in the region of the long (*a*) and short (*b*) overlapping zones (↓). The ends of an aggregate were twisted by 180° (*1* and *2*) and compared with the actual cross-striation (*)

24 h in vitro, while proliferation continues in the hand region (Merker, 1975; Neubert et al., 1974). At the beginning of the culture period (40 to 45 somite pairs) a sufficient number of cells are available for the formation of humerus- and proximal radius-/ulna anlagen (Agnish and Kochhar, 1977). Only the growth of the distal cartilage anlage can thus be disturbed by SP54® under the chosen experimental conditions. A number of publications have already reported on the effects PG and GAG exert on proliferation – and differentiation behaviour of cells (Lippman, 1968; Bernfield et al., 1973; Nevo and Dorfman, 1972; Slavkin and Greulich, 1975). It can, therefore, be concluded from the findings that highly sulfated SP54® and Arteparon® inhibit the proliferation of the early blastemal cells to such an extent that chondrogenesis in the distal regions is prevented. The actual causal mechanism of this effect is not known. As shown with physiologically sulfated GAG, these substances are bound to the cell membrane (Vogel and Kelley, 1977; Chiarugi and Vannucchi, 1976) and receptors for such substances at the cell membrane are present (Goldstein et al., 1976; Chiarugi and Vannucchi, 1976). The binding of SP54® to these receptors may influence the dynamics of the cell or the cell membrane and various secondary mechanisms.

It is not possible to attribute the alterations of the distal cartilage skeleton solely to a proliferation inhibition of the cell population which is responsible for chondrogenesis. The residual blastema is redistributed and concentrated into only one ray with additional distal cartilage cap, which indicates not only a proliferation inhibition but also an alteration of the skeleton pattern. This effect might be attributed to the action of highly sulfated substances on the cell membrane. The blastema, whose formation precedes actual chondrogenesis, is a densely packed mesenchyme with close contact among the cells. Changes in the cellular shape and various cytoplasmic components indicate a change in the surface properties of cells shortly before chondrogenesis (Gould et al., 1972), and besides positional data the blastemal cells must also communicate among each other to realize the formation of cartilage (Summerbell, 1974). Changes in the membrane properties due to disturbances in the immediate cell surrounding or even via a binding of SP54® to the membrane itself will, therefore, also affect pattern formation.

The ensuing FLS-collagen structures are stable only in the presence of SP54® and Arteparon®. When the highly sulfated GAG are removed from the culture these structures disappear almost completely within 3 days. This disintegration takes place in two ways: on the one hand collagen aggregates in the extracellular space disintegrate, starting from the ends. Here a collagenase, secreted by the chondroblasts, must become active. Moreover, the FLS-structures are also phagocytosed by the chondroblasts and are decomposed by phagolysosomes. At the same time normal filaments of 80–120 Å thickness appear which presuppose undisturbed synthesis and secretion and thus reversibility.

References

Agnish, N.D. Kochhar, D. The role of somites in the growth and early development of mouse limb buds. Develop. Biol. **56**, 174–183 (1977)

Ananthanarayanan, S. Nimni, M.E.: Collagen of rat skin and bovine articular cartilage: Their hydrodynamic properties during interactions with proteoglycans. In: Extracellular matrices in-

fluences on gene expression. (H.C. Slavkin and R.G. Greulich, eds.), pp. 311–320. New York, London: Academic Press 1975

Anderson, J.C., Labedz, R.I., Kewley, M.A.: The effect of bovine tendon glycoprotein on the formation of fibrils from collagen solution. Biochem. J. **168**, 345–351 (1977)

Aydelotte, M.B., Kochhar, D.: Development of mouse limb buds in organ culture: Chondrogenesis in the presence of a proline analog, L-azitidine-2-carboxylic acid. Develop. Biol. **28**, 191–201 (1972)

Barrach, H.-J., Rautenberg, M., Tapken, S., Neubert, D.: Some biochemical characteristics of mouse limb buds differentiating in organ culture. In: New approaches to the evaluation of abnormal embryonic development D. Neubert and H.-J. Merker, eds., pp. 114–132. Stuttgart: Georg Thieme 1975

Barrach, H.-J., Mark, K., v.d., Gay, S.: Localization of type 1 and type II collagen in developing limb buds of mammals. In: New Approaches to the Evaluation of Abnormal Embryonic Development (ed. by D. Neubert and H.-J. Merker) pp. 145–148. Stuttgart: Georg Thieme 1975

Beil, W., Furthmayr, H., Timpl, R.: Chicken antibodies to soluble rat collagen I. Immunchemistry **9**, 779–788 (1972)

Bernfield, M.R., Cohn, R.H., Banerjee, S.D.: Glycosaminoglycans and epithelial organ formation. Am. Zool. **13**, 1067–1083 (1973)

Biggers, J.D., Gwatkin, R.B.L., Heyner, S.: Growth of embryonic avian and mammalian tibiae on a relatively simple chemically defined medium. Exp. Cell Res. **25**, 41–58 (1961)

Bonucci, E., Sartori, E., Spina, M.: Osteopetrosis fetalis. Report on a case, with special reference to ultrastructure. Virchows Arch. A, Path. Anat. and Histol. **368**, 109–121 (1975)

Burton, K.: A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. **62**, 315–323 (1956)

Chapman, J.A., Armitage, P.M.: An analysis of fibrous long spacing forms of collagen. Connect. Tiss. Res. **1**, 31–37 (1972)

Chiarugi, V.P., Vannucchi, S.: Surface heparan sulfate as a control element in eucariotic cells: A working hypothesis. J. Theor. Biol. **61**, 459–475 (1976)

Clowes, A.W., Karnowsky, M.J.: Suppression by heparin of smooth muscle cell proliferation in injured arteries. Nature **265**, 625–626 (1977)

Coupar, E.H., Chesterton, C.J.: The mechanism by which heparin stimulates transcription in isolated rat liver nuclei. Eur. J. Biochem. **79**, 525–533 (1977)

Franzblau, C., Schmid, K., Faris, B., Beldekas, J., Garvin, P., Kagan, H.M., Baum, B.J.: The interaction of collagen with α_1-acid glycoprotein. Biochem. Biophys. Acta **427**, 302-314 (1976)

Gelman, R.A., Blackwell, J.: Interaction between collagen and chondroitin-6-sulfate. Connect. Tiss. Res. **2**, 31–35 (1973)

Goldstein, J.L., Basu, S.K., Brunschede, G.Y., Brown, M.S.: Release of low density lipoprotein from its cell surface receptors by sulfated glycosaminoglycans. Cell **7**, 85–95 (1976)

Gould, R.P., Day, A., Wolpert, L.: Mesenchymal condensation and cell contact in early morphogenesis of the chick limb. Exp. Cell Res. **72**, 325–336 (1972)

Hay, E.D., Meier, S.: Glycosaminoglycan synthesis by embryonic inductors: Neural tube, notochord, and lens. J. Cell Biol. **62**, 889–898 (1974)

Huang, D.: Effect of extracellular chondroitin sulfate on cultured chondrocytes. J. Cell Biol. **62**, 881–892 (1974)

Itzhaki, R.F., Gill, D.M.: A micro-biuret method for estimating proteins. Anal. Biochem. **9**, 401–410 (1964)

Jackson, D.S.: Fibrogenesis in vivo and in vitro. In: Nature and structure of collagen. (J.T. Randall, ed.), pp. 140–157. London: Butterworth 1953

Karp, G.C., Solursh, M.: Acid mucopolysaccharide metabolism, the cell surface and primary mesenchyme cell activity in the sea urchin embryo. Develop. Biol. **41**, 110–123 (1974)

Keech, M.K.: The formation of fibrils from collagen solutions. IV. Effects of mucopolysaccharides and nucleic acids: An electron microscope study. J. Biophys. Biochem. Cytol. **9**, 193–210 (1961)

Kimata, K., Okayama, M., Oohira, A., Suzuki, S.: Cytodifferentiation and proteoglycan biosynthesis. Mol. Cell Biochem. **1**, 211–223 (1973)

Kimmig, J., Kreysel, H.W.: Zur Morphologie, Biochemie und Funktion der Proteoglykane in der Dermatologie. Klin. Wschr. **51**, 207–213 (1973)

Kosher, R.A.: Inhibition of "spontaneous", notochord-induced, and collagen-induced in vitro

somite chondrogenesis by cyclic AMP derivatives and theophylline. Develop. Biol. **53**, 265–276 (1976)

Kosher, R.A., Lash, J.W.: Notochordal stimulation of in vitro somite chondrogenesis before and after enzymatic removal of perinotochordal materials. Develop. Biol. **42**, 362–378 (1975)

Kosher, R.A., Lash, J.W., Minor, R.R: Environmental enhancement of in vitro chondrogenesis. IV. Stimulation of somite chondrogenesis by exogenous chondromucoprotein. Develop. Biol. **35**, 210–220 (1973)

Kühn, K.: Die End-an-End-Verknüpfung der Tropokollagenmoleküle. Das Leder **13**, 86–93 (1962)

Kühn, K., Zimmer, E.: Eigenschaften des Tropokollagen-Moleküls und deren Bedeutung für die Fibrillenbildung. Z. Naturforsch. **16b**, 648 (1961)

Kühn, K., Grassmann, W., Hofmann, V.: Über die Bildung der Kollagenfibrillen aus gelöstem Kollagen und die Funktion der kohlenhydrathaltigen Begleitkomponenten. Z. Naturforsch. **14b**, 436–443 (1959)

Kühn, K., Kühn, J., Schuppler, G.: Kollagenfibrillen mit anormalem Querstreifungsmuster. Naturwissenschaften **51**, 337–338 (1964)

Lippman, S.M.: Glycosaminoglycans and cell division. In: Epithelial-mesenchymal interactions (R. Fleischmajer and R.E. Billingham, eds.), pp. 208–229. Baltimore: William and Wilkins Co. 1968

Lowther, D.A., Natarajan, M.: The influence of glycoprotein on collagen fibril formation in the presence of chondroitin sulphate proteoglycan. Biochem. J. **127**, 607–608 (1962)

Mark, K. von der, Gauss, V., Mark, H. von der, Müller, P.: Relationship between cell shape and type of collagen synthesized as chondrocytes lose their cartilage phenotype in culture. Nature **269**, 531–532 (1977)

Mathews, M.B.: The interaction of collagen and acid mucopolysaccharides. Biochem. J. **96**, 710–716 (1965)

Mathews, M.B., Decker, L.: The effect of acid mucopolysaccharides and acid mucopolysaccharide proteins on fibril formation from collagen solutions. Biochem. J. **109**, 517–526 (1968)

Meier, S., Hay, E.D.: Control of corneal differentiation by extracellular materials. Collagen as a promoter and stabilizer of epithelial stroma production. Develop. Biol. **38**, 249–270 (1974)

Meier, S., Heay, E.D.: Stimulation of corneal differentiation by interaction between cell surface and extracellular matrix. I. Morphometric analysis of transfilter "induction". J. Cell Biol. **66**, 275–291 (1975)

Merker, H.-J.: Significance of the limb bud culture system for investigations of teratogenic mechanisms. In: New approaches to the evaluation of abnormal embryonic development (D. Neubert and H.-J. Merker, eds.), pp. 161–199. Stuttgart: Georg Thieme 1975

Merker, H.-J., Günther, Th.: Die elektronenmikroskopische Darstellung von Glykosaminoglykanen im Gewebe mit Rutheniumrot. Histochemie **34**, 293–303 (1973)

Merker, H.-J., Toepfer, K.-H., Günther, Th.: Effect of pentosanpolysulfate SP54 on the collagen of embryonic limb buds cultured in vitro. Experentia **33**, 657–658 (1977)

Montagnier, L.: In: Growth control in cell culture (G.E.W. Wolstenholme and J. Knight, eds.), pp. 189–197. London: Churchill Livingstone 1968

Nemeth-Csoka, N.: The relationship between chemical structure and fibrogenic character of glycosaminoglycans. In: Connective tissue, biochemistry and pathophysiology (Fricke, R., Hartmann, F., eds.), pp. 61–63. Berlin-Heidelberg-New York: Springer 1974

Neubert, D., Merker, H.-J., Tapken, S.: Comparative studies on the prenatal development of mouse extremities in vivo and in organ culture. Naunyn-Schmiedeberg's Arch. Pharmacol. **286**, 251–270 (1974)

Nevo, A., Dorfman, A.: Stimulation of chondromucoprotein synthesis in chondrocytes by extracellular chondromucoprotein. Proc. Nat. Acad. Sci. USA **69**, 2069–2072 (1972)

Öbrink, B.: The influence of glycosaminoglycans on the formation of fibres from monomeric tropocollagen in vitro. Eur. J. Biochem. **34**, 129–137 (1973a)

Öbrink, B.: A study of interaction between monomeric tropocollagen and glycosaminoglycans. Eur. J. Biochem. **33**, 387–400 (1973b)

Öbrink, B., Laurent, T.C., Carlsson, B.: The binding of chondroitin sulphate to collagen. FEBS Letters **56**, 166–169 (1975)

Oegema, T.R., Laidlaw, J., Hascall, V.C., Dziewiatkowski, D.D.: The effect of proteoglycans on the formation of fibrils from collagen solution. Arch. Biochem. Biophys. **170**, 698–709 (1975)

Ogston, A.G.: The biological functions of the glycosaminoglycans. In: Chemistry and molecular biology of the intercellular matrix (E.A. Balazs, ed.), Vol. III, pp. 1231–1234. New York London: Academic Press 1970

Pratt, R.M., Larsen, M.A., Johnston, M.C.: Migration of cranial neural crest cells in a cell-free hyaluronate-rich matrix. Develop. Biol. **44**, 298–305 (1975)

Reddi, A.H., Anderson, W.A.: Collagenous bone matrix-induced endochondral ossification and hemopoiesis. J. Cell Biol. **69**, 557–572 (1976)

Rubin, H.: In: Growth control in cell culture (G.E.W. Wolstenholme and J. Knight, eds.), pp. 12–19. London: Churchill Livingstone 1968

Schaffrath, D., Stuhlsatz, H.W., Greiling, H.: Interactions of glycosaminoglycans with DNA and RNA synthesizing enzymes in vitro. Hoppe Seyler's Z. Phys. Chem. **357**, 499–508 (1976)

Schmidt, G., S.J. Thannhauser: A method for the determination of deoxyribonucleic acid, ribonucleic acid, and phosphoproteins in animal tissues. J. Biol. Chem. **161**, 83–89 (1945)

Scott, J.E.: Physiological function and chemical composition of pericellular proteoglycan (an evolutionary view). Phil. Trans. R. Soc. London B **271**, 233–410 (1975)

Seki, S., Oda, T.: Differential effects of heparin on replicative DNA synthesis and unscheduled DNA synthesis. Biochim. Biophys. Acta **479**, 391–399 (1977)

Slavkin, H.C., Greulich, R.C.: In: Extracellular matrix influences on gene expression. New York, London: Academic Press 1975

Spooner, B.S., Conrad, G.W.: The role of extracellular materials in cell movements. I. Inhibition of mucopolysaccharide synthesis does not stop ruffling membrane activity or cell movement. J. Cell Biol. **65**, 286–297 (1975)

Stegemann, H., Stadler, K.: Determination of hydroxyproline. Clin. Chim. Acta **18**, 267–273 (1967)

Strudel, G.: Control of the phenotypic vertebral cartilage differentiation by the periaxial extracellular material. In: Extracellular Matrix Influences on Gene Expression (H.C. Slavkin and R.C. Greulich, eds.), pp. 655–670. New York, London: Academic Press 1975

Strudel, G.: The primary connective tissue matrix of the bird embryo. In: Frontiers of matrix biology, 3, (L. Robert, ed.), pp. 77–100. Basel: Karger 1976

Summerbell, D.: Interaction between the proximo-distal and anteriorposterior coordinates of positional value during the specification of positional information in the early development of the chick limb bud. J. Embryol. exp. Morphol. **32**, 227–237 (1974)

Toole, B.P.: Solubility of collagen fibrils formed in vitro in the presence of sulphated acid mucopolysaccharide-protein. Nature **222**, 872–873 (1969)

Toole, B.P.: Hyaluronate and hyaluronidase in morphogenesis and differentiation. Am. Zool. **13**, 1061–1065 (1973)

Toole, B.P.: Binding and precipitation of soluble collagens by chick embryo cartilage proteoglycan. J. Biol. Chem. **251**, 895–897 (1976)

Toole, B.P., Gross, J.: The extracellular matrix of the regenerating newt limb: Synthesis and removal of hyaluronate prior to differentiation. Develop. Biol. **25**, 57–77 (1971)

Toole, B.P., Lowther, D.A.: The effect of chondroitin-sulphateprotein on the formation of collagen fibrils in vitro. Biochem. J. **109**, 857–866 (1968)

Toole, B.P., Jackson, G., Gross, J.: Hyaluronate in morphogenesis: Inhibition of chondrogenesis in vitro. Proc. Nat. Acad. Sci. USA **69**, 1384–1386 (1972)

Trowell, O.: The culture of mature organs via synthetic medium. Exp. Cell Res. **16**, 118–147 (1959)

Vogel, K.G., Kelley, R.O.: Cell surface glycosaminoglycans: Identification and organization in cultured human embryo fibroblasts. J. Cell Physiol. **92**, 469–480 (1977)

Wiebkin, O.W., Muir, H.: The effect of hyaluronic acid on proteoglycan synthesis and secretion by chondrocytes of adult cartilage. Phil. Trans. R. Soc. London B. **271**, 283–292 (1975)

Wood, G.C.: The formation of fibrils from collagen solution. 3. Effect of chondroitin sulphate and some other naturally occurring polyanions on the rate of formation. Biochem. J. **75**, 605–612 (1960)

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