THE TIBIAL GROWING CARTILAGE BIOPSY IN THE
STUDY OF GROWTH DISTURBANCES

By
V. Stănescu, C. Bona and V. Ionescu

ABSTRACT

51 biopsies of tibial growing cartilage were performed in children with
and without growth disturbances. The biopsy procedure is described.
The clinical and roentgenological follow up did not reveal any untoward
effects due to the operation. The histological and cyto-enzymological
methods used in the study of the biopsies as well as the main results
obtained are presented and discussed. In a few cases the effect of a
growth stimulating treatment could be studied by means of a repeated
biopsy from the opposite leg.

Growing cartilage has a very complex structure. Its function is based on as
yet unknown unclear intrinsic mechanisms some of which are probably similar
to the process of embryonic induction. These are influenced by extrinsic factors
such as humoral, hormonal, nutritional, nervous etc. Their effects are only
partially known and such basic phenomena as »catch up« growth, age change
response to growth hormone stimulation etc., are still unexplained. There are
differences between various species of mammals as regards the structure and
function of growing cartilage. We have previously described the histochemical
differences between growing cartilage in rats and humans (Bona et al. 1964,
1965a).

Investigations of normal and abnormal human growing cartilage are rather
scanty. This is mainly due to their being most frequently based on post mortem
examinations; biopsy material has so far been infrequently used.

Since 1960 one of us (Stănescu 1960) has been using growing cartilage biopsies
in the study of various types of growth disturbances. This was done after
a preliminary study in rabbits which demonstrated that small pieces of tibial cartilage can be removed without any disturbances of tibial development. Later on it became possible in a cooperative research to study the histopathology of the cartilage in a larger number of patients (Milcoup et al. 1962) and also to introduce histochemical and cyto-enzymological techniques in the study of several syndromes (Stănescu et al. 1965, 1966, 1967b, 1968a,b; Bona et al. 1965b, 1966, 1967a,b). These techniques were first studied in the rat epiphyseal cartilage (Bona et al. 1964).

**MATERIAL AND METHODS**

**Biopsy procedure**

The antero-medial aspect of the proximal end of the tibia was exposed sub-periosteally, the growth cartilage was identified and a small fragment centered on the cartilage line and measuring about 5 × 5 × 15 mm was removed*. The procedure was usually performed under light general anaesthesia (fluothane); local procaine anaesthesia was used in a few of the older children. Postoperative bleeding of any significance only occurred in 2 cases, but no special measures were required. 51 biopsies have so far been carried out; 32 cases were followed-up clinically and roentgenologically for a period of 1 to 6\(\frac{1}{2}\) years, the average follow-up period being 3\(\frac{1}{2}\) years.

None of the operated children showed any untoward effect of the operation such as limb inequality or deviation; nor did subsequent x-rays show any visible alterations in the size or shape of the proximal epiphysis of the tibia as compared with its previous appearance or with the opposite, unoperated side. In some cases a small radio dense area or a small osteophyte was noted at the metaphysis or the diaphysis level.

**Histological, histochemical and cyto-enzymological methods**

In a first group of patients the cartilage was studied histologically only by using trichloracetic acid decalcification and haemotoxylin-eosin and a trichromic staining.

In the second group of patients, the biopsy specimens were studied both histochemically and cyto-enzymologically. The biopsy specimen was usually divided into three fragments and processed according to the methods described in Table 1. In some cases staining for lipids or sulphated glycolipids (Holländer 1963) was also performed. In all cases special care was given to the plane of cutting the specimens so that all zones were represented.

Cyto-enzymological studies consisted in tests for the detection of the activity of the following enzymes: succinate, isocitrate, glutamate, lactate, dehydrogenase (Pearse 1961), 5-nucleotidase (Wachstein & Meisel method; Pearse 1961), ATP-ase (Wachstein & Meisel method), cysteine desulphurase (Lillie 1965), glucose-6-phosphatase (Pearse 1961), esterase (substrate: a-naphtyl acetate, Burstone 1962), acid phosphatase (azo dye, Burstone 1962), leucylaminopeptidase (Burstone 1962). In some cases y-glutamyltranspeptidase (Lillie 1965), y-galactosidase (substrate 6-bromo 2 naphtyl-y, D galactoside, Burstone 1962), glucose-6-phosphate and gluconate dehydrogenase activities

* Less than \(\frac{1}{100}\) of the irregularly shaped cartilage area.
### Table 1.
Histochemical and cyto-enzymological methods.

<table>
<thead>
<tr>
<th>Fixation</th>
<th>Decalcification</th>
<th>Stainings</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1st fragment</strong></td>
<td>Carnoy for 4 h</td>
<td><em>Histopathology:</em> haematoxylin-eosin.</td>
</tr>
<tr>
<td></td>
<td>EDTA disodium 5% in 0.1 M phosphate buffer pH 7.0*</td>
<td><em>Nucleic acids:</em> Feulgen, gallocyanin pH 1.64, methyl-green pyronine, controlled by ribonuclease (*NBC) extraction, 1 mg/ml for 3 h at 37°C.</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Collagen:</em> Van Gieson staining, controlled by collagenase (Mann) 1 h at 37°C.</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Proteins (amino-groups):</em> bromphenol blue, dinitrofluorobenzene. Some structural aminoacids: the Millon reaction, DDD.p.dimethylbenzaldehyde after Adams, tetraazotized benzidine (in some cases only).</td>
</tr>
<tr>
<td><strong>2nd fragment</strong></td>
<td>0.5% cetyltrimethylammonium bromide in ethanol formalin 9:1 for 24 h</td>
<td><em>Glycogen:</em> PAS controlled by alpha-amylase pretreatment, pyridine extraction and acetylation blocking.</td>
</tr>
<tr>
<td></td>
<td>EDTA disodium 5% in 0.1 M phosphate buffer pH 7.0*</td>
<td><em>Mucopolysaccharides and glycoproteins:</em> alcian blue 1% at pH 1 and pH 2.3; metachromasia toluidine blue 0.1% at pH 2.3 and pH 4; PAS-Hale (Mowry 1958); PAS; periodical acid diamine (Spiecer &amp; Jarles 1961).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>In some cases: barium rhodizante (Stempien 1963), bi-col staining (Wolman 1961), basic fuchsine (Stempien 1962).</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Control with:</em> a) testicular hyaluronidase (NBC) 1 mg/ml saline medium, extraction for 3 h 37°C; b) methylation-saponification; c) trypsin (NBC) 0.1 mg/ml in phosphate saline buffer 0.1 pH 8.3 for 30 min at 37°C; d) sialidase (Wellcome) 1:4 v/v in acetate buffer 0.25 M pH 5 for 24 h at 37°C.</td>
</tr>
<tr>
<td><strong>3rd fragment</strong></td>
<td>placed in ice chilled beakers and immediately put unfixed</td>
<td><em>Fresh-frozen sections after Balogh (1962)</em> followed by tests for enzymatic activities (see text).</td>
</tr>
<tr>
<td></td>
<td>EDTA disodium 10% in 0.1 M phosphate buffer pH 7.0 at 4°C*</td>
<td></td>
</tr>
</tbody>
</table>

*) Adjusted to neutral with 5 M NaOH.
were also tested (Pearse 1961). Controls were performed using specific inhibitors (Burstone 1962).

Case material

The group of cases in which the cartilage was studied histologically only included patients with pituitary dwarfism, congenital myxoedema, Turner's and Marfan's syndromes, achondroplasia, polyepiphyseal, spondylo-epiphyseal and late spondylo-epiphyseal dysplasia as well as dysplasia epiphysealis punctata. Among these cases were 3 normal children who had died instantaneously after traffic accidents. The group studied histochemically and histo-enzymologically is represented in Table 2. This group includes two children who had no growth disturbances but in whom tibial grafts were taken for orthopaedic surgical repair of osteomyelitis in the other leg; the cartilage biopsies were taken at the same time as the graft form the normal tibia. All patients had no treatment for at least 4 months and most of them had had no previous treatment. The classification of chondrodystrophies proposed by Lamy & Maroteaux (1960) was used.

RESULTS

Histopathological, histochemical and cyto-enzymological results

The data concerning the histopathology, histochemistry and cyto-enzymology in some of the syndromes studied have been presented in detail elsewhere (Stănescu et al. 1965, 1966, 1968; Bona et al. 1965b, 1966, 1967a,b). Here only some general and comparative results are presented (Table 2). Staining for mucopolysaccharides shows a certain distribution in relation to the cellular zones. The distribution is modified in many syndromes. In the normal cartilage there is an increase of metachromasia (at a low pH) in the matrix of the proliferative and upper hypertrophic areas and a disappearance of the metachromatic material in the lower part of the hypertrophic area. This zone is not only metachromatically negative but also PAS positive, alcianophilic, van Gieson pale positive and the staining for $-\text{NH}_2$ groups is stronger than in the other zones of the matrix. Patches of bi-col red coloured material are also present. In several abnormal cartilages the metachromasia extends downwards into the hypertrophic area up to the vascular zone. The persistence of metachromatic material in the lower hypertrophic area was found in Turner's syndrome, pituitary dwarfism, congenital myxoedema and de Lange's syndrome. In achondroplasia and in Morquios disease the metachromasia in the proliferative and hypertrophic areas is reduced to the perichondroplastic rims. In these syndromes many von Gieson positive bundles are present in the matrix. In polyepiphyseal dysplasia the metachromatic and Hale positive material is almost absent from the matrix, which is alcianophilic, van Gieson negative and positive for $-\text{NH}_2$ groups staining. An abundance of metachromatic and Hale positive material was found within the cells. In pseudohyppoparathyroidism, the metachromasia is intense in the perichondroplastic rims and
pericolumnar area of the basal and proliferative areas and less intense in the pericolumnar area. The lower hypertrophic zone is orthochromatic.

The results of enzymatic digestions show a partial reversal of staining for acid mucopolysaccharides by sialidase in Turner’s and de Lange’s syndrome and myxoedema. A partial effect is also found in the proliferative matrix in pseudohydroparathyroidism. In our study in the normal cartilage the metachromatic material was resistant to sialidase digestion. In Hurler’s syndrome the staining for acid mucopolysaccharides is resistant to testicular hyaluronidase digestion. In this syndrome the Holländer staining for sulphated glycolipid (controlled by hot acetone extraction) is positive in the matrix. The hot acetone extraction does not reverse the Alcian staining.

In normal cartilage the van Gieson staining is pale positive in the matrix of the hypertrophic zone and positive in the directory lines and osteoid area. The staining is decreased or negative in cartilage matrix of pituitary dwarfism, de Lange’s syndrome and polypeiphyseal dysplasia. The reaction is very marked with positive bundles in the matrix in achondroplasia and Morquio’s syndrome. Some fibrillar fuchsinophilic material is found in the basal and hypertrophic areas in Hurler’s syndrome. In congenital myxoedema fuchsinophilic material is present in the cytoplasm of chondrocytes.

In many pathological conditions, staining for glycogen and RNA in chondrocytes is weak or negative. In Turner’s syndrome staining for glycogen is apparently normal. In this condition the Feulgen staining for DNA is decreased in the nuclei of the proliferative and hypertrophic cells. In the normal cartilage, many enzymatic activities are detected (Table 2). In the abnormal cartilages various enzymatic patterns are found. Some enzymatic activities were not detected in several abnormal cartilages: e.g. alkaline phosphatase, ATP-ase, 5-nucleotidase. Other alterations were found in a single or in a few syndromes (Table 2).

**Results of growth stimulating treatment**

In a patient with acenidroplasia aged 7 we were able to obtain a cartilage biopsy in the opposite leg after treatment with anabolic steroids in a high dose for 3 months (5 mg per kg body weight, per month, Durabolin). During this period the child had grown 3 cm. The appearance of the growing cartilage was considerably changed after treatment. Before treatment the structure was desorganised with extensive fibrosis. After treatment the cartilage appeared to have an organised structure with columns and islets while the cells in the resting zone also appeared to be stimulated and arranged in groups of 2 or 4. The van Gieson staining showed absence of fibrosis in the cartilage. Acid mucopolysaccharides were more abundant and homogeneous in the treated cartilage and some enzymatic activities were stimulated. Since the bone age became markedly accelerated and virilising phenomena appeared, anabolic
Fig. 1. Achondroplasia. Hale-PAS staining. Very little Hale positive material around the chondroblasts ×125.

Fig. 2. Achondroplasia. Hale-PAS. The same patient as in Figs. 5, 6 and 7, after treatment with anabolic steroids. Abundant Hale positive material in the matrix × 125.

Fig. 3. Achondroplasia. The same patient as in Fig. 5, after treatment with anabolic steroids. Almost complete disappearance of the fibrosis × 125.

Fig. 4. Achondroplasia. Van Gieson staining. Many wide fuchsinophilic bundles in the matrix × 125.

Fig. 5. Turner’s syndrome. Toluidine blue pH 2.3. The metachromatic material extends to the vascular area × 125.

Fig. 6. Normal cartilage. Toluidine blue pH 2.3. Disappearance of metachromasia in the lower part of the hypertrophic area × 125.

Fig. 7. Hurler’s syndrome. Holländer’s staining after hot acetone extraction. The staining became negative × 125.

Fig. 8. Polyepiphyseal dysplasia. Hale-PAS staining. Absence of Hale positive material from the matrix. Rich Hale positive material within the cells × 240.

Fig. 9. Congenital myxoedema. PAS staining. Homogeneous positive matrix × 125.

Fig. 10. Turner’s syndrome. Bromphenol blue. The cartilage matrix is negative. Osteoid is positive × 125.

Fig. 11. Hurler’s syndrome. Holländer’s staining for sulphated glycolipids. Strong positive matrix × 125.

Fig. 12. Achondroplasia. Toluidine blue pH 2.3. The metachromatic material is reduced to perichondroplastic rims × 13.
### Table 2.

<table>
<thead>
<tr>
<th>Cases with normal growing cartilage 10 and 12 years old</th>
<th>Histological and histopathological findings</th>
<th>Stainings for mucopolysaccharides</th>
<th>Staining for collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal zone (about 50 per cent of the cartilage) rich in chondroblasts with 2–4–8 cells. Wide proliferative area with parallel regular and dense columns of cells, well represented hypertrophic area and degenerated chondrocytes. Deep and regular vascular penetration, long directory lines, osteoblastic pseudoepithelium, osteoclasts present.</td>
<td>Metachromic material pH 2.3 (acid mps.) - in chondrocytes: present - in the matrix basal area: around chondroplasts only; proliferating and upper hypertrophic area: intensive and uniform: lower hypertrophic area: metachromatic negative except some narrow perichondroplastic rims. Enzymatic digestion: reversed by hyaluronidase (except partially resistant perichondroplastic rims) resistant to sialidase and trypsin. Alcian weak in basal area stronger in proliferative and hypertrophic zones. PAS strong in lower hypertrophic zone. Patches of bi-col red materials in the hypertrophic matrix.</td>
<td>Positive (pale) in the matrix of the hypertrophic zone. Positive in directory lines and osteoid area.</td>
<td></td>
</tr>
</tbody>
</table>

| Pituitary dwarfism 11½/12, 11, 11⅔/12, 13½/12, 14⅔/12 years old | Narrow cartilage with relatively very large basal zone with scarce cells; short columns set well apart from one another, narrow hypertrophic area, reduced vascular penetration with short directory lines, few osteoblasts. | Metachromatic material: - present in chondrocytes in resting and proliferating areas. - In the matrix: basal area: around chondroplasts; proliferating and hypertrophic areas: homogeneously positive, extends to the vascular zone. Alcian blue similar distribution. PAS positive especially in the hypertrophic zone. Enzymatic digestions: Metachromasia and alcianophilia partially resistant to hyaluronidase, resistant to sialidase. Trypsin reverses partially metachromasia in the proliferative area. | Very pale positive in the matrix of the hypertrophic area. Positive in Howship's lacunae. |
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<th>Staining for proteins (aminogroups)</th>
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</thead>
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<tr>
<td>Positive in the cells, especially in the hypertrophic area. Weak in the matrix, stronger in the hypertrophic zone and osteoid.</td>
<td>Rich RNA content of cells in proliferative and hypertrophic areas</td>
<td>Chondrocytes with rich glycogen content especially in the hypertrophic area.</td>
<td>Alkaline and acid phosphatase present, stronger in the hypertrophic area. 5-nucleotidase, ATP-ase, succinate, malic, isocitrate dehydrogenases well represented; lactate dehydrogenase moderate; glucose-6-phosphatase; lipase and $\beta$-hydroxybutyrate dehydrogenase (especially in h. zone); $\alpha$-glycerophosphate-dehydrogenase, leucylaminopeptidase, cysteine desulphurase in basal and proliferative areas. Not detected: glucose-6-phosphate and gluconate-6-phosphate dehydrogenases.</td>
</tr>
<tr>
<td>Decreased within the cells and in the matrix.</td>
<td>Low RNA content.</td>
<td>Low glycogen content.</td>
<td>Alkaline phosphatase present in only one case of the 5 studied. Acid phosphatase present, stronger in h. area; 5-nucleotidase, ATP-ase not detected. Succinate dehydrogenase present; malic and isocitrate dehydrogenase low activity; lactate and glutamate-dehydrogenase low activity. Not detected: $\beta$-hydroxybutyrate, glucose 6 P, gluconate 6 P dehydrogenases. Detected: glucose 6 phosphatase, aminopeptidase, cysteinesulphurase, $\beta$-galactosidase, $\gamma$-glutamyltranspeptidase, esterase.</td>
</tr>
<tr>
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</tr>
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</tr>
</tbody>
</table>
| Congenital myxoedema 7, 12, 13 and 15 years old | Wide basal zone with scarce and single cells; very reduced proliferating area, somewhat better represented hypertrophic area; poor vascular penetration, lacunae often closed by osteoid rims; short directory lines. many osteoblasts | Metachromatic material:  
- present in chondrocytes;  
- intensive and homogeneously metachromatic, alcian and PAS positive matrix in all zones.  
Enzymatic digestions:  
metachromasia reversed by hyaluronidase (partial resistant perichondroplastic rims), partially reversed by sialidase, resistant to trypsin. | Fuchsinophilic material present in the cytoplasm of proliferating and hypertrophic cells. Positive in the matrix of the hypertrophic area and in the directory lines. |
| Turner's syndrome 12, 12, 16, 12 and 14 years old | Basal area with scarce cells. Proliferating area with islets and short columns of cells. Many tachychromatic or pyknotic nuclei. Relatively wide hypertrophic area, good vascular penetration, narrow directory lines. | Metachromatic material:  
- present in the cells.  
- in the matrix of all zones to the vascular area.  
Enzymatic digestion: in basal area reversed by hyaluronidase resistant to sialidase.  
In proliferating and hypertrophic zones reversed by hyaluronidases and partially by sialidase. Alcian negative in the basal, positive in the proliferating and hypertrophic areas. PAS positive in the hypertrophic matrix. | Not performed. |
| de Lange's syndrome 6 years old | Narrow cartilage with relatively large basal zone with rare, single, swollen cells. Proliferating zone contains isolated cells or more rarely islets of cells | Metachromatic material:  
- present in the cells.  
- the whole matrix is homogeneous metachromatic, metachromasia extending to the vascular zone.  
Enzymatic digestion: reversed by hyaluronidase, resistant to | Negative in the cartilage; positive in the osteoid area. |
| Table 2 (cont.) |
|-----------------|------------------|------------------|------------------|
| **Staining for proteins (aminogroups)** | **Stainings for nucleic acids** | **Staining for glycogen** | **Enzymatic tests** |
| 5 | 6 | 7 | 8 |
| Present in basal and proliferative cells; present in matrix; stronger in the hypertrophic area. | Low RNA content. | Low glycogen content. | Alkaline and acid phosphatase: low activities. 5-nucleotidase, ATP-ase, glucose-6-phosphatase: low activities; succinate dehydrogenase present; malic and isocitrate-dehydrogenases: low activities. Lactate dehydrogenase well represented. Detected: esterase, γ-glutamyl transpeptidase, β-galactosidase, sulphatase, aminopeptidase. Non-detected: β-glucuronidase. |
| Present in cells especially in the nuclei. Very weak in the matrix. | DNA staining decreased in the nuclei of the proliferative and hypertrophic area. Low RNA content of cells. | Apparently normal staining. | Alkaline phosphatase, ATP-ase, 5-nucleotidase non detected. -glucose 6 phosphatase (in one case). -acid phosphatase (in one case). -succinate, malic, isocitrate dehydrogenases present. β-hydroxybutyrate, lactic dehydrogenases weak activity; glucose-6-phosphate dehydrogenase present, weak. α-glycerophosphate-dehydrogenase not detected. -leucylaminopeptidase, very strong; cysteine desulphurase present. Non specific esterase not detected. |
| Positive in the cells. Negative in the matrix. | Low RNA content. | Low glycogen content. | Alkaline and acid phosphatase moderate; weak or moderate 5-nucleotidase, glucose-6-phosphatase, succinate dehydrogenase. Not detected: cysteine desulphurase, aminopeptidase, β-glucuronidase, γ-glutamyl transpeptidase, isocitrate dehydro- |

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Table 2 (cont.).

<table>
<thead>
<tr>
<th>Cases</th>
<th>Histological and histopathological findings</th>
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<th>Staining for collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>still disposed along the growth axis. Reduced hypertrophic area, short directory lines. The chondrocyte nuclei in all areas often pyknotic or tachychromatic.</td>
<td>trypsin; sialidase partial effect in the proliferating zone.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Alcian: perichondroplastic rims in basal area, homogeneous in proliferating and hypertrophic zones.</td>
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<tr>
<td></td>
<td></td>
<td>PAS negative in the hypertrophic matrix (except perichondroplastic rims).</td>
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</tr>
</tbody>
</table>

Hurler's syndrome 7½ years old

Narrow cartilage, narrow basal zone, short columns and islets of enlarged cells in the proliferating area; narrow hypertrophic zone, very short directory lines.

Metachromatic material:
- rich in chondrocytes.
- the whole matrix metachromatic up to the vascular zone.
Enzymatic digestion: resistant to hyaluronidase. Alcian: positive matrix, becomes stronger after hyaluronidase. PAS positive reaction around chondroplasts and in directory lines. Bi-col material: hyaluronidase sensitive, in the hypertrophic matrix.
The Holländer staining for sulphated glycolipids positive in the whole matrix, reversed by hot acetone extraction. The same extraction has no effect on alcian positive material.

Achondroplasia 9 and 11 years old

Relatively wide resting zone with scarce single cells. Distended proliferative cells with degenerative changes, without columnar arrangement, isolated or in small islets.

Metachromatic material:
- only in the cell of the resting zone. In the matrix:
- present in the matrix of the basal zone.
- in the matrix of the proliferating and hypertrophic area present only in the perichondroplastic rims.

Fibrous bundles strongly positive in the matrix.
<table>
<thead>
<tr>
<th>Staining for proteins (aminogroups)</th>
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<th>Enzymatic tests</th>
</tr>
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<tbody>
<tr>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
</tr>
</tbody>
</table>

- **Genase.**
- Strong reactions for glutamate dehydrogenase and lactate dehydrogenase activities.

| Positive in the cytoplasm of chondrocytes (except the hypertrophic area). The matrix and the directory lines intense staining with bromphenol blue and ninhydrin; pale with dinitrofluorobenzene. | Low RNA content. | Low glycogen content. | Alkaline phosphatase, ATP-ase, 5-nucleotidase, acid phosphatase present; glucose-6-phosphatase weak. -succinate, malic and isocitrate dehydrogenases present. The last two less strong. -aminopeptidase strong, γ-glutamylyltranspeptidase weak, sulphatase present. Not detected in the chondrocytes: β-galactosidase, cysteine desulphurase. |
| Positive in the basal cells. Positive in the basal matrix, very strong in the fibrovascular band. In the matrix of proliferating and hypertrophic areas, | Low RNA content in proliferative and hypertrophic areas. | No glycogen in proliferative and hypertrophic areas. | Alkaline-phosphatase, ATP-ase, not detected. -5-nucleotidase, glucose-6-phosphatase. -α-glycerophosphate dehydrogenase: very weak. -succinate dehydrogenase present. -malic, isocitrate, lactic dehydrogenases, esterase weak. |
Table 2 (cont.).

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<tr>
<td>1</td>
<td>Fibrous bundles in the matrix parallel to the cartilage axis. Lack of directory lines in many places. The fibrotic cartilage seems to merge directly into lamellar bone. Many vessels in the bone cartilage matrix. In one case a transverse connective-vascular band was present between the basal and proliferative area.</td>
<td>Enzymatic extraction: hyaluronidase sensitive, sialidase and trypsin resistant. Alcian: similar to metachromasia. PAS positive material in the matrix of the proliferating and hypertrophic zone; bi-col positive material in the hypertrophic zone.</td>
<td>Fibrous bundles positive in the whole matrix. Fuchsinophilic material around some chondrocytes.</td>
</tr>
<tr>
<td></td>
<td><strong>Morquio’s disease</strong>&lt;br&gt;10 years old (distal femoral cartilage)</td>
<td>Disordered pattern of cells; pyknotic and vacuolated cells, the zones can not be identified. Fibrous bundles in the matrix, irregularly disposed. No directory lines, the fibrotic cartilage seems to merge into lamellar bone.</td>
<td>Metachromatic material: present in cells, few and scattered in the matrix especially around chondroplasts. The larger part of the matrix fibrotic and negative. Enzymatic extraction: reversed by hyaluronidase. Alcian: similar to metachromasia. PAS positive near the bone. Bi-col positive material in the fibrotic zone.</td>
</tr>
<tr>
<td></td>
<td><strong>Polyepiphyseal dysplasia</strong>&lt;br&gt;12½/12, 15, 11½/12 and 7 years old</td>
<td>Basal zone representing about 50 per cent of the cartilage. Proliferating zone with relatively short columns or islets of cells. Large chondrocytes in hypertrophic</td>
<td>Metachromatic material: Very abundant in the chondrocytes of the proliferating and hypertrophic zone. Negative in the matrix. Matrix: alcian and PAS positive, resistant to hyaluronidase. Absence of red bi-col material.</td>
</tr>
</tbody>
</table>

590
Table 2 (cont.).

<table>
<thead>
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<td>6</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>very strong in the fibrotic zones.</td>
<td>-leucylaminopeptidase, acid phosphatase present.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Low in the cells, Low RNA content. Absent or very low. Alkaline phosphatase, ATP-ase, 5-nucleotidase, α-glycerophosphate dehydrogenase, β-galactosidase, β-glucuronidase, β-hydroxybutyrate and glutamate dehydrogenases: not detected. Lactate dehydrogenase, esterase, succinate, malic, isocitrate, dehydrogenases: low activities; acid phosphatase, aminopeptidase cysteine desulphurase: moderate to low; γ-glutamyl transpeptidase relatively strong.

Positive in the cells. Strongly positive in the matrix. Low RNA content. Low glycogen content. Alkaline phosphatase not detected. ATP-ase, glucose-6-phosphatase weak. 5-nucleotidase very strong. succinate, malic, isocitrate dehydrogenases present. lactate dehydrogenase weak. α-glycerophosphate, β-hydroxy-
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<td>1</td>
<td>zone, good vascular penetration, long directory lines.</td>
<td>Metachromatic material:</td>
<td>Not performed.</td>
</tr>
</tbody>
</table>

Pseudo-hypoparathyroidism: 

- Nine girls, 9-12 years old. 
- Narrow cartilage, relatively wide basal area, short columns of cells. Extremely long directory lines. No osteoclasts detected.
- Enzymatic digestion: reversed by hyaluronidase. Resistant to sialidase, partial effect in the proliferative matrix. Unusual response to trypsin: sensitive material in the proliferating matrix; after treatment hypertrophic area and directory lines become metachromatic.
- Alcian: positive perichondroplastic in basal and proliferating area, faintly positive in the remainder or the matrix. Strong reaction in the hypertrophic area and directory lines. PAS positive especially in the hypertrophic matrix.
**Table 2 (cont.).**

<table>
<thead>
<tr>
<th>Staining for proteins (aminogroups)</th>
<th>Stainings for nucleic acids</th>
<th>Staining for glycogen</th>
<th>Enzymatic tests</th>
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<td>5</td>
<td>6</td>
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<td>8</td>
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- Butyrate dehydrogenases very low.
- Esterase moderate.
- Not detected: glutamate dehydrogenase, β-galactosidase, β-glucuronidase.
- Cysteine desulphurase weak;
- Aminopeptidase strong only in proliferating area;
- Acid phosphatase weak.

- Not performed.
- Not performed.
- Apparently normal staining for glycogen.

- Alkaline phosphatase present.
- ATP-ase, 5-nucleotidase moderate;
- γ-glutamyltranspeptidase, esterase, acid phosphatase, glucose-6-phosphatase: low activities;
- Lactate and glutamate dehydrogenases: strong activities.
- Not detected: isocitrate dehydrogenase, β-glucuronidase, cysteine desulphurase, leucylaminopeptidase.
- Succinate dehydrogenase moderate.
steroids cannot be considered as a means of treatment for achondroplasia. However, it is interesting that this greatly altered cartilage is capable of responding to stimulation with a non specific treatment (Stănescu et al. 1968).

In another patient with achondroplasia aged 11, the biopsy was repeated after treatment for 3 months with human growth hormone (10 mg three times a week, 1 mg = 0.8 IU). No visible alterations in the cartilage were noted and the child had grown 1 cm.

In a case of congenital myxoedema, the cartilage appeared almost normal after a three months period of treatment with thyroid extract.

**DISCUSSION**

The histopathological, histochemical and cyto-enzymological data of the epiphyseal cartilage obtained by open biopsy in children with bone and growth disorders might contribute to the understanding of the pathophysiology of the growing cartilage and of the pathogenesis of the syndromes studied. However, the interpretations of the data is rendered difficult by the fact that the complex and intricate mechanisms of the normal cartilage are still poorly understood. The limits of the histochemical methods must also be taken into account in the possible interpretations of the findings.

In principle an initial disorder of the cartilage might exert a qualitative or quantitative influence on the proliferation, differentiation and/or secretory activities of the chondrocytes by interfering with the collagen, non-collagenous proteins, various mucopolysaccharides or glycoproteins. The disorder might originate in different zones; certain asynchronisms in the secretion of these components might also occur. In other cases the initial disorder might involve the composition of body fluids, the invading capillaries, the accompanying cells, the osteoprogenitor cells, osteoblasts or osteoclasts. In some conditions several processes might be primarily disturbed. Even by using multiple investigation methods it would probably prove difficult to demonstrate which are the initial disorders and which are the consequence of the former.

The limitations of histochemical methods, especially of those for mucopolysaccharides (mps.) are well known (Meyer 1966; Zerlotti & Yaeger 1967). We tried to improve their specificity by using enzymatic digestions and by applying several tests. However, these methods are mainly qualitative and enable only the identification of reactive sites of large groups of substances. Losses and other substances present within the tissues may intervene to modify the reactions (e. g. proteins and mps. stainings). Quantitative mitochemical methods for mps. (Antonopoulos et al. 1964; Gardell & Szirmay 1967; Hjertquist 1964; Saito et al. 1968; Yamagata et al. 1968) could provide more precise information at the level of tissue structure.
Nevertheless the altered distribution of stainings for mps. in the chondrocytes and in the matrix of some abnormal cartilages as well as the changes in staining for collagen and proteins might suggest some possible basic mechanisms and some criteria for a provisional classification of the abnormal human cartilages. Some clues regarding the pathophysiology of certain syndromes might also be obtained. In the normal cartilage we found an increase of metachromasia in the matrix of the proliferative and upper hypertrophic areas and a disappearance of metachromatic material in the lower part of the hypertrophic area (Bona et al. 1965a). A similar histochemical evolution of the matrix has been reported in young rats by Sylvén (1947). Histochernical evidence has long indicated changes in the ground substance in the calcifying area (lit. in Graumann & Neumann 1964). Various explanations have been advanced for the disappearance of metachromasia: decrease in concentration of acid mps., depolymerisation, change in the protein polysaccharides complexes, appearance of a qualitatively different compound (lit. in Graumann & Neumann 1964; Zerlotti & Yaeger 1967; Bowness 1968). Studies using other methods have also suggested that calcification in cartilage is associated with a change in the ground substance. Immediately prior to calcification in the rat and calf there is a loss of staining for protein polysaccharides by immunofluorescence techniques (Hirschman & Dziewiatkowski 1966). In chick embryos electron microscopic studies a reduction in the size of protein polysaccharides granules was found in the matrix of calcified cartilage as compared to non-calcified cartilage (Matukas & Krikos 1968). Staining of fresh, unfrozen sections of rat tibia in toluidine blue demonstrated a change in the calcified cartilage which was, however, different from that found by the usual histochemical methods (Hirschman 1967). Chemical analysis and sulphate uptake studies reveal that the sulphate mps. content of bone is very much lower than that of the epiphyseal cartilage but results are conflicting as regards the exact location of the drop (lit. in Bowness 1968). Hjertquist (1964) found little difference between the mps. of the cartilage zones in the dog. Lindenbaum & Kuettner (1967) even found an increase of total mps. but small differences in the composition of protein polysaccharides in the »ossifying« zone of the calf scapula. Greer et al. (1968) in rabbits found the highest sulphate uptake and a lower hexosamine content in the hypertrophic area of the epiphyseal cartilage. However, the calcification probably takes place in a very narrow layer and this is a relatively small proportion of the total zone in which mps. have been analysed in some studies (Lindenbaum & Kuettner 1967). In our study on human normal cartilage the lower hypertrophic area is not only metachromatically negative (after EDTA decalcification and at a low pH) but also PAS positive, alcianophilic, van Gieson positive (collagen). The staining for -NH₂ groups is stronger than in the other zones of the matrix. This might suggest a higher collagen, protein and/or glycoprotein content interfering with the
mps. metachromasia. Patches of bi-col red coloured material (hyaluronates?) are also present. Hjertquist (1964) found that a small fraction of mps. probably hyaluronic acid was higher in the zone of provisional calcification than in any other zone.

An importance feature found in several abnormal cartilages is the extension of the staining for strong acid mps. to the vascular zone. The persistence of low pH metachromatic material in the lower hypertrophic area was found in Turner’s syndrome, pituitary dwarfism, myxoedema, de Lange’s and Hurler’s syndromes and may play a part in the growth disturbance. The acid mps. have an important role in the calcification mechanisms. Conflicting hypotheses suggesting a stimulating or an inhibitory role in calcification have been proposed (lit. in Fleisch 1966; Bachra 1967; Bowness 1968). It is more probable that different fractions of mps. protein complexes exist in separate compartments promoting or inhibiting calcification (lit. in Bowness 1968). Other possible functions of the normal acid mps. of this border zone between cartilage and bone might be considered: induction and stimulation effects, re-utilization of resorbed mps. and collagen in bone formation.

The presence of this alteration in a variety of syndromes suggests that it might be produced by various mechanisms. Weak staining for proteins in the hypertrophic matrix in Turner’s syndrome and high activity of some proteolytic enzymes in cartilage (Stănescu et al. 1965) and muscles (Stănescu et al. 1967a) suggest an alteration of mps. proteins complexes by excessive proteolysis. The weak staining for proteins and collagen in pituitary dwarfism rather suggests an alteration of the complexes by a deficient synthesis of the protein moicety and of collagen. This synthesis might be even more impaired than the synthesis of mps. In vitro studies on the incorporation of radioactivity from labelled substrate into the proteins and sulphated mps. of the matrix of cartilage of hypophysectomised rats have demonstrated an inhibition of incorporation (Johnston & Deiss 1965).

Abnormal mps. are demonstrated in the cartilage of Hurler’s syndrome by the resistance of the staining for acid mps. to testicular hyaluronidase digestion. In this syndrome the mps. excreted in excessive amounts in the urine are chondroitin sulphate-B and heparan monosulphate. These hyaluronidase resistant mps. have also been recovered from tissues of patients with thus disease, their relative proportions in different tissues varying considerably. Chondroitin sulphate-B and questionable heparan sulphate have been isolated from cartilage and bone, organs in which these polysaccharides are normally absent (Meyer & Hoffman 1961). In our study staining for sulphated glycolipids was positive in the cartilage of Hurler’s syndrome. Several studies demonstrated accumulation of both chondroitin sulphate and glycolipids in cases of Hurler’s syndrome (lit. in Clausen et al. 1967) but there are no data on cartilage glycolipids. The investigation of cartilage mps. and glycolipids might
be useful for the study of conditions in which probable alterations of various enzymes which participate in the degradation of mps. and/or glycolipids. Suggestions concerning the role of the lipids in normal cartilage (Wuthier & Irving 1964) might be obtained from further studies on patients with these syndromes. In congenital myxoedema mps. stain strongly and are homogeneous with a variety of stains despite greatly decreased tests for enzymatic activities. These features and data from the literature on the metabolism of mps. in hypothyroidism (Schiller et al. 1962) suggest an accumulation due to a low turnover with a very decreased break-down. The vascular penetration and resorption are greatly impaired. This has also been found by Wegelin (1926) in human myxoedema.

Another abnormal finding in congenital myxoedema is the positive van Gieson staining within the proliferating and hypertrophic chondrocytes. Juva et al. (1966) have produced an accumulation of protocollagen within embryonic chondrocytes by inhibiting proline hydroxylation.

In pseudohypoparathyroidism the distribution of staining for mps. is almost similar to the normal but the results of trypsin extraction suggest some abnormalities of the polysaccharides protein complexes. Very long directory lines and an absence of osteoclasts have been observed indicating disturbance in the remodelling of the primary bone.

In our study sialidase extraction did not alter the staining for acid mps. in the normal cartilage. A partial reversal of the staining was found in Turner's and de Lange's syndromes, pseudohypoparathyroidism and myxoedema. Sialic acid was found in bone (Herring 1964) and in cartilage (Anderson 1962) but the nature of the majority of glycoproteins in cartilage is as yet poorly defined (Herring 1968). The sialidase digestion was not performed in polyepiphyseal dysplasia in which the histochemical tests suggest a rich glycoprotein content of the matrix.

In achondroplasia and Morquio's syndrome there are many bundles of connective fibres in the cartilage matrix. The content of matrix acid mps. is limited to the perichondroplastic rims. The fibrosis of the cartilage might be due to abnormal mps. The collagen fibres formed from solution are influenced by mps. (Mathews 1965) and it has been suggested that mps. might represent extracellular codes for the organization of collagen (Meyer 1963). Some factors producing alteration of tropocollagen or increasing the crossing of collagen might be involved. The differentiation of mesenchymal cells in chondrocytes might also be altered. In some experiments the environment in which the cells are situated (oxygen concentration, pressure) largely determined whether the mesenchymal cells had produced or destroyed bone, cartilage or fibrous tissue (Goldhaber 1963; Shaw & Bassett 1964). As regards Morquio's syndrome a disturbance in the metabolism of mps. with a high excretion of keratosulphate in the urine is reported (Pedrini et al. 1962). In achondroplasia a special role
of the vasculo-fibrous band in some cases separating the basal from the proliferative area has been assumed by many authors (lit. in Dietrich 1939). This band has been considered as a prolongation of the periosteum or as an abnormal persistance of the »cartilage medullary channels«.

In serial sections Dietrich (1939) found fibrous bundles prolonging the transverse band to the vascular zone. Langeskiold (1947) described a fibrous periosteal type of ossification which was unable to produce a normal growth of long bones. In our study fibrous bundles parallel to the cartilage axis were found in the matrix of the proliferative and hypertrophic areas and vessels and fibrocytes were also present. In one case the transverse connective band was also found. Treatment with anabolic steroids produces an almost complete disappearance of the fibrosis and an increase of the acid mps. staining. This suggests that the stimulated chondrocytes secrete mps. capable of reversing the fibrosis of the cartilage. Another anabolic hormone, human growth hormone has apparently no effect on the acondroplastic chondrocytes. It is known that the effects of androgens and growth hormone on the morphology of normal cartilage and on bone age are different. In Hurler’s syndrome a fibrillar faintly fuchsinophilic structure was found in the matrix. In our biopsy fragment the large fibrotic bands found by Meyer (1963) in cartilage and bone were not observed.

In polyepiphyseal dysplasia the staining for acid mps. and collagen are almost negative in the matrix and an abundant material positive for acid mps. staining is found within the cells. A disorder in the »merocrine« secretory process of the protein mps. complexes might be present in this syndrome. The matrix displays positive staining for proteins and alcianophilia suggesting a glycoprotein content. A decreased growth with bone formation showing roentgenologically a good mineralisation and clinically a good resistance is thus possible in epiphyseal plates which are poor in metachromic material (histochemical detectable strong acid mps.).

Staining for glycogen is positive in the normal cartilage especially in the hypertrophic area, whereas many abnormal cartilages have a poor glycogen content (Table 2). The relations between glycogenolysis and calcification in cartilage have been reviewed (Gutman & Yu 1949). Glycogen was considered as a source of energy as well as a precursor of matrix components and phosphates.

In normal cartilage the Brachet technique for RNA disclosed a rich content especially in the hypertrophic area, in agreement with the autoradiographic electron microscope studies (Godman & Porter 1960; Koburg 1962). A poor content in this area was found in many abnormal cartilages indicating a decreased synthetic activity. As regards DNA tests a decreased staining was observed in the proliferative and hypertrophic area in the cartilage in Turner’s syndrome but this must be verified by quantitative methods.
The enzymatic activities were studied after EDTA decalcification which probably produces some alterations. This type of decalcification, however, preserves many enzymatic activities in the cartilage (Balogh 1962; Mori et al. 1965; Takada 1966a,b). In normal cartilage the enzymes involved in the Krebs cycle that were investigated were well represented.

The microsomal enzyme glucose-6-phosphatase and the 5-nucleotidase were detected in accordance with the rough endoplasmic reticulum and the high RNA content of the chondrocytes particularly of the hypertrophic zone (Godman & Porter 1960; Bona et al. 1965a). The ATP-ase and alkaline phosphatase enzymes participating in the calcification process were well represented especially in the hypertrophic zone. The lysosomal enzymes studied were also detected (acid phosphatase, leucyl-aminopeptidase). The activity of γ-glycerophosphate dehydrogenase was detected in the resting and proliferating areas. No activity of enzymes involved in the first 2 steps of the pentosic shunt could be detected. Lactate dehydrogenase was well represented.

Various enzymatic patterns were noted in the abnormal cartilage. Some enzymatic activities were not detected in several abnormal cartilages: alkaline phosphatase, ATP-ase and 5-nucleotidase. The alteration of other tests seems to point to some more specific enzymatic disorder. Thus a low β-galactosidase activity in Hurler’s syndrome was also found in the skin and the liver of these patients and it was suggested that this deficiency might explain the accumulation of both mps. and glycolipids (van Gemund 1968; Ockerman 1967; Ockerman & Köhlin 1968). The high activity of some proteolytic enzymes in Turner’s syndrome was also detected in the muscle (Stănescu et al. 1967a). The absence of isocitrate dehydrogenase in pseudohypoparathyroidism might explain the resistance to parathyroid hormone co-existing in some cases with bone lesions similar to those found in hyperparathyroidism etc. It was not possible to study enzymes with an important role in the synthesis of mps. such as UDPG-dehydrogenase, glutamine-fructose-6-phosphate aminotransferase etc.

The use of a variety of methods for the study of abnormal human cartilage biopsies would probably lead to a better understanding of human growth disturbances.

REFERENCES

Bona C., Pitis M., Stănescu V. & Ionescu V.: Acta histochem. (Jena) 18 (1964) 295.
Bona C., Stânescu V., Dimitrescu M., Ghyka G. & Ionescu V.: Acta histochem. (Jena) 21 (1965a) 98.
Bona C., Stânescu V. & Ionescu V.: Acta histochem. (Jena) 21 (1965b) 284.
Bona C., Stânescu V., Dimitrescu M. & Ionescu V.: Acta histochem. (Jena) 27 (1967a) 207.
Bona C., Stânescu V., Streja D. & Ionescu V.: Acta histochem. (Jena) 23 (1966) 231
Koburg E.: Ann. histochem. 7 (1962) 97.
Stănescu V., Bona C. & Ionescu V.: Acta histochem. (Jena) 30 (1968a) 1.
Stempien M.: J. Histochem. Cytochem. 10 (1962) 84.

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